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(54) Title: PREPARATION OF RECOMBINANT BORRELIA PROTEINS

(57) Abstract

Highly-purified immunologically-effected recombinant protein encoded by a gene of a full-length wild-type *Borrelia* lipoprotein, particularly the OspA encoded by the *OspA* gene of *B.burgdorferi*, useful in vaccines and test kits for Lyme disease, is prepared by inducing protein production from a host organism transformed by a plasmid vector containing the cloned gene and subsequently recovering and purifying the protein. The grown organism is lysed and contacted with a surfactant, particularly Triton X-114, which selectively extracts the desired protein from the lysed cells. Upon heating the resulting mixture to mildly-elevated temperature, a detergent phase separates out and is recovered separate from the other phases. The detergent phase is further purified from residual protein by column chromatography.

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TITLE OF INVENTION

PREPARATION OF RECOMBINANT BORRELIA PROTEINS

FIELD OF INVENTION

The present invention relates to the preparation of recombinant Borrelia lipoproteins, particularly the outer surface protein A (OspA) of Borrelia burgdorferi, the spirochete responsible for Lyme disease.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States patent application Serial No. 888,765 filed May 27, 1992, which itself is a continuation-in-part of copending United States patent application Serial No. 779,048 filed October 18, 1991.

BACKGROUND TO THE INVENTION

Lyme disease is a zoonosis caused by the tick-borne spirochete Borrelia burgdorferi. The spirochete can cause serious dermatological, arthritic, neurological and other pathological disorders in an infected host. Recently Lyme disease has become a serious epidemiological concern, particularly in North America, but also elsewhere in the world.

Attempts are underway to develop a vaccine against the disease and immunodiagnostic reagents useful in the detection of antibodies against the spirochete. Much attention has focused on the major outer surface protein OspA, antibodies against which have been demonstrated to provide protection against challenge with the spirochete in mice (refs. 1, 2 - identification of the literature references appears at the end of the disclosure).

Previous attempts have been made to isolate purified, soluble Borrelia lipoproteins through the growth and subsequent purification of Borrelia cell cultures. However, the growth and subsequent purification of the proteins from crude cell extracts of Borrelia is very time consuming and expensive.

Recombinant techniques have been suggested for producing OspA and derivatives thereof, in view of the potential for production of large quantities of pure protein material. Such techniques would involve expression of Borrelia genes in a suitable host/vector expression system, such as E. coli.

Published International (PCT) Patent application No. WO 90/04411 describes a DNA fragment encoding the OspA protein of B. burgdorferi of the New York strain B31 (ATCC 35210) and containing the ospA gene. The nucleotide sequence of the ospA gene, coding for the full-length wild-type OspA, is described in the published PCT patent application, along with the derived amino-acid sequence.

Dunn et al have described the preparation of a modified form of OspA protein which is soluble, yet retains specific reactivity to antibodies against wild-type B. Burgdorferi OspA (ref. 3). The paper describes the preparation of two plasmids, pET9-OspA and pET9-preOspA, the former containing a polymerase chain reaction (PCR)-amplified DNA sequence coding for a recombinant truncated version of OspA, and the latter containing a PCR-amplified DNA sequence coding for the full-length, wild-type OspA. Both sequences are expressed from the bacteriophage T7 ϕ 10 promoter.

The primary translation product of the full-length gene contains a hydrophobic N-terminal leader sequence, which is a substrate for the attachment of lipid moiety to the sulfhydryl side chain of the adjacent cysteine residue. Following this attachment, cleavage by signal peptidase II and the attachment of lipid moieties to the new N-terminus occurs. On the other hand, the protein translated from the truncated gene is not lipidated, and is soluble in aqueous solution. Expression of the soluble, truncated derivative of OspA is said to overcome certain problems said to be associated with expressing

recombinant versions of the full-length wild-type Borrelia burgdorferi lipoproteins using E. coli, namely that the protein has poor solubility properties, due to the association of the protein with the outer cell membrane of the host during expression, requiring the use of detergents to effect separation of the protein and difficult purification procedures.

The plasmids were transferred to an expression strain of E. coli, namely BL21(DE3)(pLysS) (a host strain containing a chromosomal copy of the gene for T7 RNA polymerase under control of the inducible lacUV5 promoter and a pACYC184 based plasmid pLysS, which produces low levels of T7 lysozyme). Upon induction, plasmid pET9-preOspA was found to produce relatively smaller amounts of inducible protein than plasmid pET9-OspA. The latter product was found to be soluble in the absence of detergent while the former required treatment with the detergent Triton X-100 to solubilize. The Dunn et al paper contains no description of any subsequent isolation and purification of the detergent-solubilized OspA protein.

Although production of recombinant full-length OspA may be achieved in lower quantities than the soluble truncated variation of OspA and the use of detergents is necessary for recovery of the full-length protein (treatment of lipoproteins with detergents often impairs reactivity), nevertheless production of the recombinant wild-type protein is considered desirable, since the lipidated wild-type protein is thought to produce a greater immune response than the truncated protein, due to the stimulation of both B lymphocytes (refs. 4, 5) and cytotoxic T lymphocytes (ref. 6) by lipid covalently attached to the N-terminus of a peptide in an identical fashion to that in which lipid moiety is attached to B. burgdorferi OspA (ref. 7).

Long-lasting protective immunity to spirochetal challenge has been produced in mice by vaccination with OspA (refs. 1, 8). The antigen used in these studies was a fusion protein containing a large domain of glutathione-S-transferase at the amino terminus of OspA. This fusion protein is unlikely to be a substrate for a post-translational lipid attachment. The ability of this antigen to induce a protective response in these studies may be due to the fact that the antigen was delivered in Freund's complete adjuvant, with multiple secondary doses in Freund's incomplete adjuvant (refs. 1, 8), or in the form of live or killed whole E. coli expressing the fusion protein (ref. 9). With respect to this prior activity, reference also is made to published International application WO 92/00055.

As will be seen herein, it has now been shown that the recombinant protein encoded by the full length ospA gene, in unadjuvanted or alum-absorbed form, exhibits an immunogenicity which is not shown by the corresponding recombinant OspA protein lacking the attached lipid. This difference in immunogenicity has been shown not to be due to any difference between the antigenicity of the two proteins. The present invention represents the first disclosure of which the inventors are aware of enhancement of the immunogenicity of a large protein antigen by expression of a bacterial lipoprotein.

SUMMARY OF INVENTION

The present invention provides, in one aspect, a novel, simple and effective manner of producing a highly-purified immunologically-effective recombinant protein encoded by the full-length wild-type B. burgdorferi ospA gene and other full-length Borrelia lipoproteins encoded by the respective genes. The recombinant protein exhibits an immunogenicity which is equal to that of the native protein.

The present invention employs selective separation of the recombinant lipoprotein from other cellular constituents using particular detergents. The procedure of the present invention enables the perceived prior art problems associated with production of the full-length OspA protein to be overcome, while providing a product having enhanced immunogenicity.

For the first time, there is provided by the present invention a highly-purified immunologically-effective recombinant protein which is encoded by a full-length wild-type Borrelia lipoprotein gene, particularly the B.burgdorferi ospA gene, and which is formed recombinantly from a host organism transformed by a plasmid containing the gene. Such novel protein forms a further aspect of the present invention. Certain novel plasmids, useful in such transformation, also are provided herein.

The novel protein product provided herein is useful in vaccines against infection by Borrelia organisms, particularly those causing Lyme disease, and such vaccines, comprising an immunologically-effective amount of the protein, particularly that encoded by the full-length wild-type B. burgdorferi ospA gene, constitute an additional aspect of the invention.

In a yet further aspect of the invention, there is provided a method of immunization of a mammal against infection by Borrelia organisms, particularly those causing Lyme disease, by administering an immunologically-effective amount of the protein, particularly that encoded by the full-length wild type B. burgdorferi ospA gene, in the absence of adjuvant.

The novel proteins also may be used as immunodiagnostic agents for the detection of antibodies against infection of a host by a Borrelia organism, particularly that causing Lyme disease, and hence the presence of such infection.

There have been produced herein, as described below, purified immunologically-effective recombinant protein encoded by the full-length ospA gene from several different specific strains of B. burgdorferi, specifically the B31, ACA1 and Ip90 strains. There also exist full-length ospA genes which differ in nucleotide sequence from the sequences of these specific genes by at the most a few nucleic acids, resulting in gene products which differ at most by a few amino acids from those produced by the actual B31, ACA1 and Ip90 strain ospA genes. Such genes and gene products are encomposed herein within the B31, ACA1 and Ip90 families of strains.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the PCR oligonucleotides used in cloning the B-31, ACA1 and Ip90 full-length ospA gene of B. burgdorferi into the pET9a expression vector;

Figure 2 illustrates the cloning strategy for inserting the full-length ospA gene into the pET9a expression vector so as place the ospA gene under control of the T7 ϕ 10 promoter, to form pOA1 from the B31 gene, pOA9 from the ACA1 gene and pOA10 from the Ip90 gene;

Figure 3 is a predicted restriction map of plasmid pOA1;

Figure 4 shows the results of various restriction digests of plasmid pOA1, demonstrating that all predicted sites are present (M= markers (ϕX 174 DNA digested with Hae III); B= Bam HI; E= Eco RI; H= Hind III; N= Nde I);

Figure 5 shows the PCR nucleotides used in cloning the B-31, ACA1 and Ip90 full-length ospA gene of B. burgdorferi into the pCMB1 expression vector;

Figure 6 illustrates the cloning strategy for inserting the full-length ospA gene into the pCMB1 expression vector, so as to place the ospA gene under the control of the Trc promoter, to form pOA5 from the B31 gene, pOA7 from the ACA1 gene and pOA8 from the Ip90 gene;

Figures 7A, 7B and 7C show a time course of induction with ITPG of OspA in two host strains containing pOA1 (Figure 7A), and a host strain containing pOA5 (Figure 7B) and pOA6 (Figure 7C);

Figures 8A, 8B and 8C are flow charts showing, respectively, the cell growth and lysis, the detergent extraction and the purification steps involved in the production and purification of recombinant full-length OspA from E. coli in accordance with one embodiment of the invention;

Figure 9 shows the solubilization of proteins in whole cell lysate with various detergents (Deo= sodium deoxycholate; Emp= Empigen; Sarc= sodium lauryl sarcosinate; SDS= sodium dodecyl sulfate; TX-114= Triton X-114), with the lanes being (M= markers (Bio-Rad Low Molecular Weight Standards); W= whole lysate; S= soluble fraction; P= pellet; A= aqueous phase; D= detergent);

Figure 10 is an immunoblot with the anti-OspA monoclonal antibody H5332, showing that the 31 Kilodalton species in the detergent phase is OspA, Wh= whole cell lysate, Aq= aqueous phase after Triton X-114 extraction, Dt= detergent phase, Bx= aqueous phase back extracted with Triton X-114, Pl= insoluble pellet;

Figure 11 illustrates Triton X-114 phase partitioning of lipoprotein and non-lipoprotein for pOA1 and pOA2, in which bacteria containing pOA1 expressing OspA lipoprotein or pOA2 expressing OspA non-lipoprotein were grown to mid-log phase and induced with ITPG for 2 hours for pOA1 and 4 hours for pOA2. After centrifugation, cells were resuspended and lysis was effected by freezing and thawing. Triton X-114 was added and phase partitioning was produced by heating to 37°C. An equal aliquot of each fraction was electrophoresed on 4 to 20% SDS-PAGE gel, and the gel was stained with Coomassie Brilliant Blue. The fractions are molecular weight markers (M), whole lysate

(W), aqueous phase (A), detergent phase (D), and insoluble pellet (P) for pOA1 and pOA2 as indicated;

Figures 12A, 12B and 12C illustrate purification of OspA lipoprotein produced from pOA1 (Figure 12A), from pOA9 and pOA10 (Figure 12B) and from pOA5, pOA7 and pOA8 (Figure 12 C), where an aliquot of each fraction was electrophoresed on a 4 to 20% SDS-PAGE gel and the gel was stained with Coomassie Brilliant Blue. Figure 12A : lane 1, low molecular weight markers; lane 2, whole lysate; lane 3, detergent phase; lane 4, DEAE-Sepharose; and lane 5, S-Sepharose elution; Figure 12B : P= prestained low molecular weight markers (106 kDa, 80kDa, 49.5kDa, 32.5kDa, 27.5kDa, 18.5kDa), CL= whole cell lysate, D=Triton X114 detergent phase, De= DEAE Sepharose flow through fraction. S= Sephrose, pH5.7 elution (purified OspA-2); Figure 12C : P=prestained low molecular weight markers (106kDa, 80kDa, 49.5kDa, 32.5kDa, 27.5kDa, 18.5kDa), O= B31 OspA-L S-Sepharose purified, derived from pOA1 (Figure 12A), CL= whole cell lysate, D= Triton X114 detergent phase, De=DEAE Sepharose flow through fraction.

Figure 13 is a silver-stained gel of three test vaccines used in a study comparing the immunogenicity of the purified full-length OspA to that of the protein from Borrelia burgdorferi (M= markers; OA= purified recombinant OspA; E= B. burgdorferi fraction E; SA= bovine serum albumin);

Figure 14 shows the IgG serum titres of mice given a boost four weeks after primary injection with 10 μg of the purified full-length OspA and B. burgdorferi fraction of Figure 13, with an ELISA assay being performed with 100 ng purified OspA in the solid phase, mouse serum as the first antibody and goat anti-mouse IgG conjugated to alkaline phosphatase as the second antibody;

Figures 15A to 15E contain a graphical depiction of the dose response of mice to lipoprotein. Figure 15A

shows the dose response of C3H/He mice to unadsorbed lipoprotein; Figure 15B shows the dose response of BALB/c mice to unadsorbed lipoprotein; Figure 15C shows the dose response of C3H/He mice to lipoprotein adsorbed to alum; Figure 15D shows the dose response of CH3/He mice to unadsorbed lipoprotein; Figure 15E shows the dose response CH3/He mice to non-lipidated protein. Mice were vaccinated at week 0 with the indicated amount of protein, in PBS except where noted, and boosted with the same vaccine at week 3. Serum IgG titre at week 4 was determined by ELISA using OspA lipoprotein as antigen;

Figure 16 contains a graphical depiction of the antigenicity of the lipoprotein and non-lipoprotein; and

Figure 17 illustrates the production of plasmids pCMB1 and pCMB2.

IDENTIFICATION OF ospA GENE-CONTAINING PLASMIDS

Plasmid I.D.	B. burgdorferi Strain	Expression Vector
pOA1	B31	pET9a
pOA2*	B31	pET9a
pOA5	B31	pCMB1
pOA6	B31	pCMB2
pOA7	ACA1	pCMB1
pOA8	Ip90	pCMB1
pOA9	ACA1	pET9a
pOA10	Ip90	pET9a

* This plasmid contains truncated ospA of B31 strain. All other plasmids contain full-length ospA of various strains.

GENERAL DESCRIPTION OF INVENTION

The cloned ospA gene of B. burgdorferi strain B31 (as described in the above-mentioned WO 90/04411) (N-terminal region : SEQ ID No: 1, C-terminal region : SEQ ID No: 2. The remainder of the sequence is shown in WO 90/04411) was used as a template (pTRH44) and specially-designed

oligonucleotide primers (PET-IN [CO1] (SEQ ID No: 3) and PET-273C [CO3] (SEQ ID No: 4)) were used in a polymerase chain reaction (PCR) to amplify the whole of the wild-type ospA gene, as shown in Figure 1.

Similarly, the cloned ospA gene of B. burgdorferi strains ACA1 and Ip90 (as described in reference 10 - N-terminal region of ACA1 and Ip90 is: SEQ ID No: 1; C-terminal region of ACA1: SEQ ID No: 5; C-terminal region of Ip90: SEQ ID No: 6) was used in a PCR reaction with oligonucleotide primer pairs (a) OspN2 (SEQ ID No: 7) and BZ1 (SEQ ID No: 8) and (b) OspN 2 (SEQ ID No: 7) and pK4 (SEQ ID No: 9), respectively at the N- and C-terminal ends to form the appropriate amplified fragments, as shown in Figure 1.

The basic methods for amplifying a desired target nucleic acid sequence using oligonucleotide primers are generally known in the art and are described in U.S. Patents No. 4,683,202 and 4,800,159. Reference may be had to such patents for description of the techniques to be employed.

The resulting fragments were cloned into the NdeI and Bam HI sites of the plasmid vector pET9 to place the ospA gene under control of a T7 promoter and efficient translation initiation signals from bacteriophage T7, as seen in Figure 2. The pET9 and pLysS plasmids, the bacterial hosts for cloning, growth media and the methods used to direct expression of cloned genes by T7 RNA polymerase have previously been described in U.S. Patent No. 4,952,496 and reference may be had thereto for such description. While a T7 promoter system is one preferred expression system in the present invention, expression of the full-length ospA gene may be achieved utilizing other expression systems compatible with the host organism, as described below.

The pET9 expression vector was used since it has a kan gene as its selective marker rather than a bla gene. Consequently, ampicillin is not used during cell growth and hence there is no possibility that an immunogenic ampicilloyl/OspA target protein conjugate can be formed. Such conjugates are believed to be major antigenic determinants in penicillin allergy and may complicate immunological studies.

The resulting plasmids have been designated pOA1, pOA9 and pOA10, containing the ospA genes from B31, ACA1 and Ip90 strains of B. burgdorferi respectively. The pOA1 plasmid is nearly identical to the pET9-preOspA plasmid described by Dunn et al (supra), except that the oligonucleotides used for the PCR reaction were different in the two cases. A predicted restriction map for the plasmid pOA1 is shown in Figure 3, while Figure 4 contains the results of various restriction digests of plasmid pOA1, demonstrating that all the predicted sites are present.

For protein production, the plasmids pOA1, pOA9 and pOA10 were transformed into the expression strain of E. coli or other suitable host organism. Preferably, the E. coli strain is the T7 expression strain of E. coli, as described in the aforementioned U.S. Patent No. 4,952,496. Specifically, the strain may be the expression strain BL21(DE3)(pLysS) of E. coli, as described above, or E. coli strain HMS174(DE3)(pLysS). The transformed host was grown and protein was induced with isopropyl-ß-D-thiogalactoside (IPTG). A time course of induction of OspA from plasmid pOA1, following IPTG addition, is shown in Figure 7A. Identical results to those for pOA1 were obtained using pOA9 and pOA10. Synthesis of OspA protein from plasmid pOA1 ceased approximately one hour after induction, implying some toxicity of the protein to E.

coli. Nevertheless, the protein production was at an acceptable level of approximately 10 mg/L of cell culture.

In addition to the provision of plasmids pOA1, pOA9 and pOA10 and expression of OspA lipoprotein in E. coli using the T7 promoter, further plasmids have been constructed containing the full-length B31, ACA1 and Ip90 ospA gene under a different promoter and expression of lipoprotein has been achieved. In this regard, plasmids pOA5 and pOA6 were prepared by cloning a PCR-amplified fragment of ospA from B31 strain into the NcoI and Bam HI sites of plasmid expression vectors pCMB1 and pCMB2 while plasmids pOA7 and pOA8 were prepared by cloning a PCR-amplified fragment of OspA from ACA1 strain (pOA7) and from Ip90 strain (pOA8) into the NcoI and Bam HI sites of expression vector pCMB1 (see Figure 6 for pOA5, pOA7 and pOA8).

As seen in Figure 5, the cloned ospA genes of B. burgdorferi strains B31, ACA1 and Ip90 were amplified by PCR reaction using oligonucleotide primer pairs (a) PK3 (SEQ ID No: 10) and CO3 (SEQ ID No: 3), (b) PK3 and (SEQ ID No: 10) and BZ1 (SEQ ID No: 8), and (c) PK3 (SEQ ID No: 10) and PK4 (SEQ ID No: 9), respectively at the N- and C-terminal ends of the respective genes to form the appropriate amplified fragments.

Plasmids pCMB1 and pCMB2 were constructed by digesting plasmid pTrc99a (Pharmacia Catalog No. 27-5007-01) and a Kanamycin resistance gene (Pharmacia Catalog No. 27-4897-01), isolating the resulting fragments by the Geneclean procedure, and ligating the fragments together (Figure 17). Plasmid pTrc99a, 4197 bp, contains a strong promoter adjacent to a multiple cloning site, followed by a strong transcription termination signal (rrnB). Expression of the target gene uses the host cell RNA polymerase, allowing its use in a wide variety of E. coli strains. Expression is tightly controlled by the lactose

suppressor gene (lacIq) included on the vector. The lactose repressor protein prevents transcription of the target gene in the absence of the inducer IPTG. The kanamycin resistance gene is a linear double stranded 1282 bp DNA fragment containing the gene from the transposon Tn903 flanked by restriction enzyme sites and encodes the enzyme aminoglycoside 3'-phosphotransferase, which confers resistance to kanamycin and neomycin.

pCMB1, 5.5 kb, contains the kanamycin resistance gene oriented such that its transcription is in the same direction as that originating from the Trc promoter while pCMB2 contains the kanamycin resistance gene oriented in the opposite direction, such that transcription of the resistance gene and the gene of interest under the control of the Trc promoter result in converging transcripts. Restriction enzyme digests of pCMB1 and pCMB2 using SmaI, HindIII and BamHI+NcoI showed the exact size fragments predicted.

The plasmids pOA5 and pOA6 were transformed into the expression strain of E. coli or other suitable organism, preferably the DH5α competent cells. The transformed hosts were grown and protein was induced with IPTG. The time course of induction with pOA5 (Figure 7B) was similar to that for pOA1 (Figure 7A) while the levels of OspA produced by pOA6 (Figure 7C) was several times lower. Identical results to those for pOA5 were obtained using pOA7 and pOA8.

The steps involved in the production and purification of the recombinant full-length OspA are shown schematically in Figures 8A to 8C. Specific process conditions are recited therein as set forth in the Examples below.

Following the cell growth step and induction of protein (Figure 8A), the cells are subjected to freeze-thaw lysis. The lysate is treated with a detergent which

is selective for OspA protein solubilization, in preference to other bacterial proteins present in the lysate. A series of experiments was conducted employing different detergents to determine which detergent was selective for OspA protein. Of those tested Triton X-114 was found to selectively solubilize a 31 kilodalton protein (Figure 9), which was shown to be OspA by immunoblotting (Figure 10).

The invention is not limited to the employment of Triton X-114 but clearly also includes other materials exhibiting a similar selective solubility for OspA as well as the phase separation property under mild conditions referred to below.

Following addition of the selective detergent, the mixture is warmed to a mild temperature elevation of about 35° to 40°C at which time the solution becomes cloudy as phase separation occurs. It is essential to the purification procedure of the present invention that such phase separation occurs under mild conditions to avoid any denaturing or other impairment of the immunological properties of the protein. (For example, if Triton X-100, mentioned by Dunn et al (ref. 3), is used as the detergent, while selective separation of OspA protein is effected, much higher temperatures, about 60 to 65°C, are required to effect phase separation, which is highly disadvantageous with respect to the utility of the product).

Centrifugation of the cloudy mixture results in separation of the mixture into three phases, namely a detergent phase containing 50% or more of the OspA protein and a small amount (approximately 5 wt%) of the bacterial proteins, an aqueous phase containing the balance of the bacterial proteins and a solid pellet of cell residue. The detergent phase is separated from the aqueous phase and solid pellet.

By the steps of treatment with a selective detergent for OspA and subsequent phase separation of the detergent phase, substantially complete separation of OspA from bacterial proteins is achieved (Figure 12). There remains the final purification of OspA from residual bacterial protein present in the detergent phase.

Final purification of the protein is effected on a chromatography column selective for binding bacterial proteins but not OspA, specifically DEAE-Sephacel, DEAE-Sepharose, or other equivalent chromatography material. The detergent phase is loaded onto the column and the flow-through, which contains all the purified OspA protein is collected. The bound fraction contains all the bacterial proteins in the detergent phase. Following further purification using S-Sepharose or equivalent chromatographic column (Figure 12), in addition to being free from contaminating proteins, the flow-through fraction is substantially free from liposaccharide (LPS) as indicated by lack of pyrogenicity, as determined by limulus amebocyte lysate (LAL). The highly purified solution of OspA may be freeze dried or otherwise processed.

Although the procedure described above has been specifically directed to production of highly-purified recombinant OspA protein, the procedures and techniques described are readily adaptable to the production of other highly-purified recombinant Borrelia lipoproteins, by suitable cloning of the appropriate gene, construction of a suitable plasmid vector containing the gene, transformation of a suitable host by the plasmid vector, and production and purification of the lipoprotein, by suitable choice of selective surfactant.

EXAMPLES

Example 1

Plasmid pOA1 was prepared as described above and used to transform *E. coli* strains BL21(DE3)(pLysS)(pOA1) and HMS174(DE3)(pLysS)(pOA1). The transformed *E. coli* was inoculated into LB media with 25 μg/ml of kanamycin sulfate and 25 μg/ml of chloramphenicol at a rate of 12 ml of culture for every liter prepped. The culture was grown overnight in a flask shaker at about 37°C.

The next morning, 10 ml of overnight culture medium was transferred to 1L of LB media containing 25 μg/ml of kanamycin sulfate and the culture was grown in a flask shaker at about 37°C to a level of OD=0.6 (although growth up to OD=1.5 can be effected), in approximately 3 hours.

To the culture medium was added isopropylthiogalactoside (IPTG) to a final concentration of 0.5mM and the culture medium was grown for a further two hours at about 37°C. At the end of this period, the culture medium was cooled to about 4°C and centrifuged at 10000 xg for 10 minutes. The supernatant was discarded while the cell pellet was resuspended in 1/10 the volume of PBS. The cell suspension was frozen in liquid nitrogen and may be stored indefinitely at -70°C, if desired.

Following freezing of the cell suspension, the cells were thawed to room temperature (about 20° to 25°C) which causes the cells to lyse. DNase I was added to the thawed material to a concentration of 1 μg/ml and the mixture was incubated for 30 minutes at room temperature, which resulted in a decrease in the viscosity of the material.

The incubated material was chilled on ice to a temperature below 10°C and Triton X-114 was added as a 10 wt% stock solution, to a final concentration of 0.3 to 1 wt %. The mixture was kept on ice for 20 minutes. The chilled mixture next was heated to about 37°C and held at that temperature for 10 minutes.

The solution turned very cloudy as phase separation occurred. The cloudy mixture then was centrifuged at about 20°C for 10 minutes at 12,000 xg, which caused separation of the mixture into a lower detergent phase, an upper clear aqueous phase and a solid pellet. The detergent phase was separated from the other two phases and cooled to 4°C, without disturbing the pellet. Buffer A, namely 50 mM Tris, pH 7.5, 2 mM EDTA and 10 mM NaCl, was added to the cooled detergent phase to reconstitute back to 1/3rd the original volume. The resulting solution may be frozen and stored for later processing as described below or may be immediately subjected to such processing.

A DEAE-Sepharose CL-6B column was prepared in a volume of 1 ml/10 ml of detergent phase and was washed with 2 volumes of Buffer C, namely 50 mM Tris pH 7.5, 2 mM EDTA, 1 M NaCl, 0.3 wt % Triton X-100, and then with 4 volumes of Buffer B, namely 50 mM Tris pH 7.5, 2 mM EDTA, 0.3 wt % Triton X-100.

The detergent phase then was loaded onto the column and the flow-through containing the OspA, was collected. The column was washed with 1 volume of Buffer B and the flow-through again was collected. The combined flow-through was an aqueous solution of purified OspA, which may be frozen for storage.

The column may be freed from bacterial proteins for reuse by eluting with 2 volumes of Buffer C.

Further and final purification of the flow-through from the DEAE-Sepharose column by chromatography on S-Sepharose Fast Flow. The flow-through from the DEAE-Sepharose column first was acidified to pH 4.2 by the addition of 0.1 M citric acid. The S-Sepharose Fast Flow column was washed extensively with Buffer C, adjusted to pH 4.2 with citric acid.

Highly-purified OspA was eluted from the column using Buffer C, adjusted to pH 5.7 with citric acid. The eluate

was immediately adjusted back to pH 7.5 by the addition of 2 M Tris base.

The aqueous solution of highly purified OspA obtained by both chromatography procedures was analyzed by Coomassie stained gels (Figure 12A) and confirmed to contain OspA in highly purified form. The purity of the product produced by the latter chromatography procedure was greater than that formed by the former chromatography procedure, exhibiting very low levels of endotoxin.

Example 2

The procedure of Example 1 was repeated, except that other surfactants were used as well as Triton X-114 to form the detergent phase. The results which were obtained are shown in Figure 9. As may be seen from Figure 9, only Triton X-114 of the detergents tested was able to provide a sufficiently selective solubilization of OspA to permit ready purification by column chromatography.

Example 3

Plasmids pOA5 and pOA6 were prepared as described above and used to transform E. coli strain DH5α. The protein expression procedure employed in Example 1 was repeated and the time course expression of the OspA lipoprotein by pOA5 was identical to that observed for pOA1 while OspA expression by pOA6 was found to be several times lower than by pOA5 (Figures 7B and 7C). Purification of the OspA protein was continued in pOA5-expressing cells only.

The B31 OspA lipoprotein produced by the Trc expression system in this way was purified following the identical procedure to that described in Example 1, with the exception that 0.1 mg/ml of lysozyme was added to the cell pellet after harvesting and the cells were suspended for 30 minutes at room temperature prior to freezing.

The DEAE-Sepharose column flow through contained OspA lipoprotein in a highly purified form (Figure 9C).

Example 4

The procedures for production of plasmid pOA1 (pET promoter) and pOA5 (TRC promoter) were repeated, as described above, with the cloned ospA gene of Asian strain (Ip90) of B.burgdorferi (ATCC _____), to form plasmids pOA8 (Trc promoter) and pOA10 (pET promoter) containing the gene. A restriction digest was performed on these plasmids, which showed all predicted sites to be present.

The procedures also were repeated with the cloned ospA gene of the European strain (ACA1) of B.burgdorferi (ATCC _____), to form plasmids pOA7 (Trc promoter) and pOA9 (pET promoter) containing the gene. A restriction digest was performed on these plasmids, which showed all predicted sites to be present.

Growth and induction of the pOA7 and pOA8 expression strains proceeded identically to the pOA5 strain (Example 3) while growth and induction of the pOA9 and pOA10 expression strains proceeded identically to the pOA1 strain (Example 1).

The ACA1 and Ip90 OspA lipoproteins obtained by these operations were purified identically to the B31 (pOA1) OspA lipoprotein as described in Example 1. The DEAE-Sepharose column flow through contained OspA lipoprotein in a highly purified form (Figures 12B, 12C).

Example 5

The full-length recombinant OspA purified from E. coli in accordance with the procedure described in Example 1 was compared with the B. burgdorferi fraction E, which is enriched for OspA (prepared as described in the aforementioned PCT No. WO 90/04411), and with bovine serum albumin (BSA) for immunogenicity in mice. C3H/He mice were injected with 10 μg of each vaccine (Figure 13) in unadjuvanted form. Four weeks after primary injection, the mice were given a boost of the same antigen as used in the primary injection. The serum titres were assayed by

an ELISA against recombinant, purified OspA lipoprotein (Figure 14).

As may be seen, both immunogens elicited a very similar response, that is a weak but detectable primary serum IgG response, with as much as a 100-fold increase in serum titres following the boost. For both immunogens, serum IgG titres remained stable for at least 6 weeks. In neither case was any significant serum IgM response observed.

These experiments indicate that the recombinant OspA is equally immunogenic as the protein purified from B. burgdorferi.

The immunological integrity of the recombinant OspA lipoprotein was further demonstrated by the fact that sera from mice immunized with the recombinant lipoprotein were fully capable of inhibiting the growth of B. burgdorferi strain B31 spirochetes in vitro, as seen from the following Table 1:

TABLE 1. Titres of B. Burgdorferi growth-inhibiting activities in sera from mice vaccinated with OspA lipoprotein and non-lipoprotein

Mouse Strain	Antigen	Dose (μg)	B. burgdorferi Growth-inhibiting Titer
C3H/He	OspA lipoprotein	0	<8
		0.1	32
		0.5	256
		2.5	1024
	OspA non-lipoprotein	0.1	<8
		0.5	<8
		2.5	<8
BALB/c	OspA lipoprotein	0	<8
		0.1	4
		0.5	16
		2.5	512
	OspA non-lipoprotein	0.1	<8
		0.5	<8
		2.5	<8

As may be seen from this Table, the inhibitory activity of the serum was found to be proportional to the dose of the lipoprotein administered to mice and correlated very well with the results obtained by the ELISA assay for anti-OspA IgG. Serum from mice vaccinated with OspA non-lipoprotein, which contained no detectable anti-OspA antibodies, had no effect on spirochete growth.

The growth inhibitory titres achieved in mice immunized with 2.5 μg of OspA lipoprotein in PBS were equal to or greater than those obtained in mice vaccinated with 20 μg of total B. burgdorferi protein in Freund's complete adjuvant and boosted with total protein in PBS. The ability of the recombinant OspA lipoprotien to elicit a serum response capable of inhibiting spirochete growth demonstrates that the critical protective epitopes on the protein are conserved during expression in E. coli and subsequent purification.

Example 6

Plasmid pOA2 was prepared in similar manner to that described above for plasmid pOA1, except that primer PET-18N, having the sequence (SEQ ID No. 11):

5' CAG CAT ATG GCT AAG CAA AAT GTT AGC 3'

was used together with the C-terminal primer PET-273C, in the PCR reaction to amplify the truncated form of the ospA gene lacking the lipoprotein signal peptide. The underlined region in PET-18N is identical to nucleotides 51 to 67 of the coding strand of the OspA gene.

The plasmid pOA2 was used to transform E. coli strains BL21 (DE3) (pLysS) (pOA2) and HMS174 (DE3) (pLysS) (pOA2) and the truncated OspA protein was expressed from the transformed E. coli, as described in Example 1. Upon performing the cell lysis and detergent extraction steps of Example 1, the non-lipidated protein was found to be present in the aqueous phase, as would be expected.

Example 7

The effect of lipid attachment to the OspA protein on immunogenicity was investigated.

C3H/He and BALB/c mice were vaccinated with either the lipoprotein form of OspA formed and isolated as described in Example 1 or the non-lipoprotein form of OspA prepared by Dunn et al (ref. 3) and boosted three weeks later. Sera were collected 1 to 2 weeks following boosting, and assayed by an ELISA using purified OspA lipoprotein as the antigen.

Figures 15A and 15B show that OspA lipoprotein in PBS induced a strong dose-dependent, secondary IgG response in both C3H/He (Figure 15A) and BALB/c (Figure 15B) mice. Little, if any, increase in immunogenicity of the lipoprotein was observed when the protein was absorbed to aluminum hydroxide at a ratio of protein: aluminum of 1:5 by weight prior to injection, as seen from Figure 15C.

In constrast to the response seen for the OspA lipoprotein, the non-lipoprotein was unable to induce any detectable anti-OspA antibody, even at the highest dose of 2.5 μg/mouse, as seen in Figure 15E. Even alum-absorbed non-lipoprotein appeared incapable of producing an immune response.

It should be observed that the lipoprotein and non-lipoprotein versions of OspA are indentical in deduced amino acid sequence except for the amino terminal residue which is modified cysteine for the lipoprotein and methionine-alanine for the non-lipoprotein, which strongly suggests that the observed difference in immunogenicity is due to the presence of the lipid moiety.

Example 8

The inherent antigenicity of the lipidated and non-lipidated OspA also was investigated, to determine whether the difference in immunogenicity observed in Example 7 was due to differences in their inherent antigenicity or to

differences in how the immune system recognizes and responds to the two proteins.

Sera from mice immunized with the lipoprotein gave a positive signal in an ELISA using either version of OspA as the test antigen, as seen in Figure 16. Similarily, sera from mice immunized with the non-lipoprotein failed to react with either antigen. Accordingly, while both versions of OspA are equally antigenic, the lipoprotein is recognized well by the immune system while the non-lipoprotein is not.

In addition, these results strongly suggest that, while the lipid group is essential for immunogenicity, the antibodies made against the lipoprotein are directed principally against the peptide portion of the molecule, rather than the lipid moiety.

Example 9

The effect of strain-source for OspA protein on immunogenicity was observed.

Mice were vaccinated with purified OspA lipoproteins B31, ACA1 and Ip90 derived from pOA1, pOA9, pOA10 respectively. The mice were vaccinated with 2.5 μg of protein at week 0, boosted with the same dose at week 3, and bled out at week 4. Serum IgG titres at week 4 were determined by an ELISA assay using purified B31, ACA1 or Ip90 OspA lipoprotein as the antigen. The titre was determined using the secondary antibody goat anti-mouse IgG.

The results obtained in these experiments are shown in Figure 15D. As may be seen from this data, recombinant OspA for all the strains tested is immunogenic in mice and polyclonal sera show a partial cross-reactivity.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel and simple procedure to produce a highly-purified immunologically-effective recombinant protein encoded by a full-length wild-type Borrelia lipoprotein gene, particularly the ospA gene of B.burgdorferi, by the use of a detergent to selectively extract the protein from the host strain and subsequent purification of the detergent solution by column chromatography. Modifications are possible within the scope of this invention.

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SEQUENCE IDENTIFICATION

SEQ ID NO.	DESCRIPTION OF SEQUENCE	FIGURE
1	Coding strand for N-terminal region of ospA from B31 strain, ACA1 and 1p90 strain	1,5
2	Coding strand for C-terminal region of ospA from B31 strain	1,5
3	Oligonucleotide primer PET-IN [CO1]	1
4	Oligonucleotide primer PET-273C [CO3]	1,5
5	Coding strand for C-Terminal region of ospA from ACA1	1,5
6	Coding strand for C-terminal region of ospA from Ip90	1,5
7	Olignucleotide primer OspN2	1
8	Olignucleotide primer BZ1	1,5
9	Olignucleotide primer PK4	1,5
10	Olignucleotide primer PK3	5
11	Olignucleotide primer pET-18N	-

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATATATTATG AAAAAATATT TATTGGG
27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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27

(3) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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26

(4) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(5) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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31

(6) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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27

(7) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(8) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(9) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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24

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(11) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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27

CLAIMS

What we claim is:

1. A pure immunologically-effective protein which is encoded by a full-length wild-type Borrelia lipoprotein gene and which is formed recombinantly from a host organism transformed by a plasmid containing the gene.

2. The protein of claim 1 which is encoded by the full-length wild-type B. burgdorferi ospA gene.

3. The protein of claim 2 wherein said plasmid is plasmid pOA1.

4. The protein of claim 2 wherein said plasmid is plasmid pOA5.

5. The protein of claim 2 wherein said plasmid is plasmid pOA6.

6. The protein of claim 2 wherein said plasmid is plasmid pOA7.

7. The protein of claim 2 wherein said plasmid is plasmid pOA8.

8. The protein of claim 2 wherein said plasmid is plasmid pOA9.

9. The protein of claim 2 wherein said plasmid is plasmid pOA10.

10. The protein of claim 2 wherein said ospA gene is that isolated from the B31 family of strains of B. burgdorferi.

11. The protein of claim 2 wherein said ospA gene is that isolated from the Ip90 family of strains of B. burgdorferi.

12. The protein of claim 2 wherein said ospA gene is that isolated from ACA1 family of strains of B. burgdorferi.

13. The protein of claim 1 wherein said host organism is E. coli.

14. The protein of claim 2 wherein said plasmid comprises the plasmid vector pET9 in which the gene is placed under the control of a T7 promoter and efficient translation initiation signals from bacteriophage T7.

15. The protein of claim 14 wherein said host organism is a T7 expression strain of E. coli.

16. The protein of claim 15 wherein said host organism is the BL21(DE3)(pLysS) or the HMS174(DE3)(pLysS) strain of E. coli.

17. The protein of claim 2 wherein said plasmid comprises the plasmid vector pCMB1 or pCMB2 in which the gene is placed under the control of a Trc promoter.

18. The protein of claim 1 which is substantially free from bacterial protein and from lipopolysaccharide.

19. A process for the production of a protein encoded by a full-length wild-type Borrelia lipoprotein gene, which comprises:

effecting induction of Borrelia lipoprotein from a host organism transformed by a plasmid containing said gene,

lysing the cells of said host organism,

treating the lysed cells with a surfactant which selectively solubilizes Borrelia lipoprotein in preference to bacterial and other proteins and which is able to effect phase separation of a detergent phase under mild conditions,

effecting phase separation into a detergent phase containing solubilized Borrelia lipoprotein, an aqueous phase containing bacterial and other proteins and a solid phase containing cell residue,

recovering said detergent phase from said solid phase and said aqueous phase, and

purifying said detergent phase free from proteins other than Borrelia lipoprotein.

20. The process of claim 19 wherein the protein is OspA protein encoded by the ospA gene of B.burgdorferi.

21. The process of claim 20 wherein said surfactant is Triton X-114.

22. The process of claim 21 wherein said treating of said lysed cells is effected at a temperature of about 0° to about 10°C, the resulting mixture is heated to a mildly-elevated temperature of about 35° to about 40°C to effect separation of said detergent phase, and said detergent phase is separated from said aqueous phase and said solid phase by centrifugation.

23. The process of claim 22 wherein said purification of said detergent phase is effected by contacting said detergent phase with a chromatography column under conditions which result in binding of proteins other than the Borrelia lipoprotein to said column and recovering the flow-through from said column containing the Borrelia lipoprotein.

24. The process of claim 23 wherein said column is further contacted with a buffer medium which displaces liquid containing Borrelia lipoprotein from said column while other proteins are retained by said column and the flow-through from said further contact is collected.

25. The process of claim 19 wherein said host organism lysis is effected by freezing and thawing the host organism.

26. The process of claim 25 wherein said host organism is a strain of E. coli.

27. The process of claim 26 wherein said plasmid is plasmid pOA1, pOA5, pOA7, pOA8, pOA9 or pOA10.

28. A vaccine against infection by Borrelia organism, comprising an immunologically-effective amount of a protein as claimed in claim 1.

29. A vaccine against infection by Lyme disease, comprising an immunologically-effective amount of a protein as claimed in claim 2.

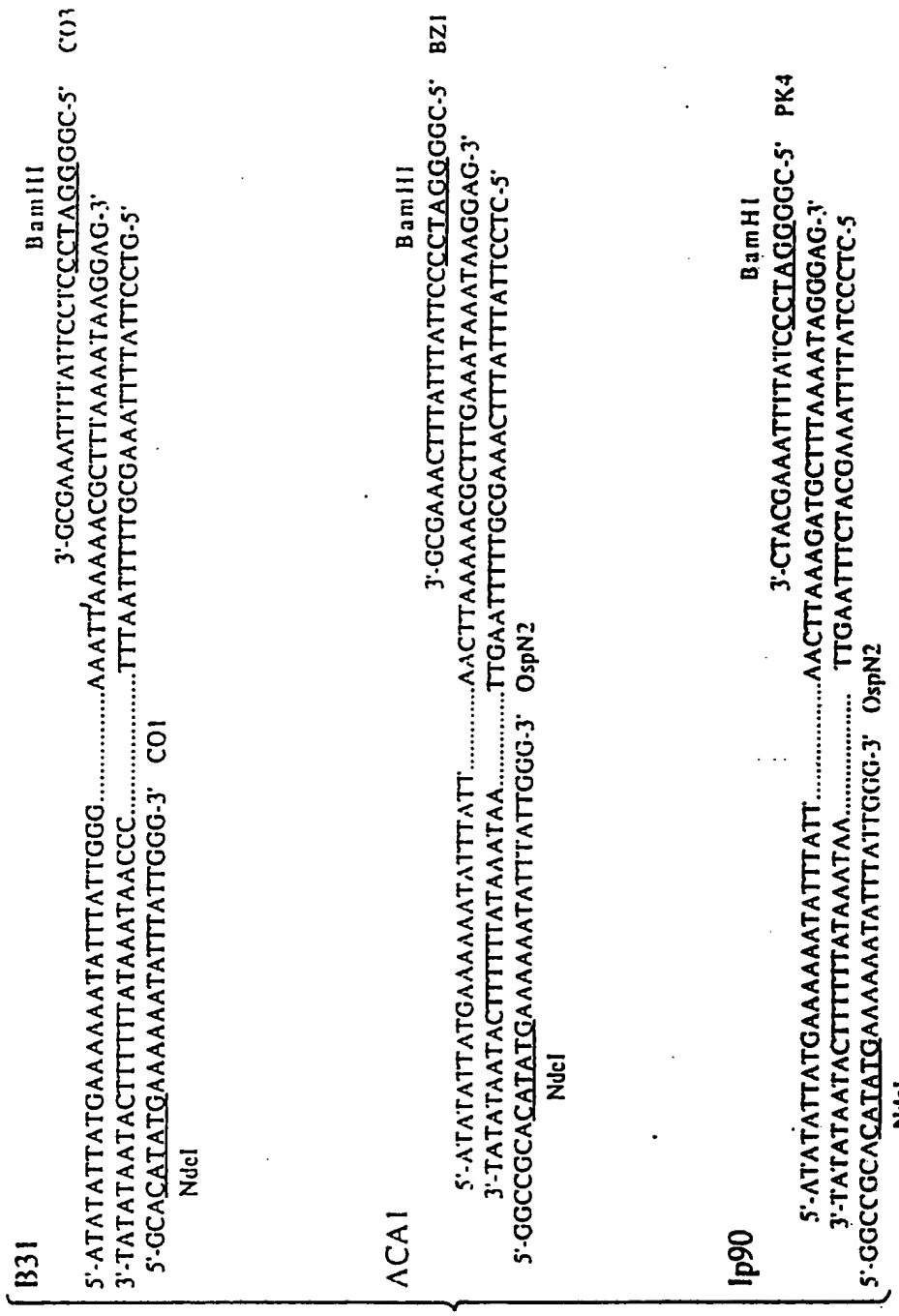
30. An immunodiagnostic agent for detection of antibodies against infection of a host by a Borrelia organism, comprising a protein as claimed in claim 1.

31. An immunodiagnostic agent for the detection of antibodies against infection of a host by Lyme disease, comprising a protein as claimed in claim 2.

32. A method of immunization of a host mammal against infection by a Borrelia organism, which comprises administering to the host an immunologically-effective amount of a protein as claimed in claim 1.

33. The method of claim 32 wherein said Borrelia organism is one causing Lyme disease and said protein is one as claimed in claim 2.

34. The method of claim 33 wherein said host mammal is a human and said protein is administered in the absence of an immunogenicity-enhancing adjuvant.


B31
BamHI
3'-GCGAAATTTTATTCCTCCTAGGGGC-5' C03
5'-ATATATTATGAAAAAATATTTATTGGG.....AAATTAAAAACGCTTTAAAATAAGGAG-3'
3'-TATATAATACTTTTTTATAAATAACCC.....TTTAATTTTTGCGAAATTTTATTCCTG-5'
5'-GCACATATGAAAAAATATTTATTGGG-3' C01
NdeI
ACA1
BamHI
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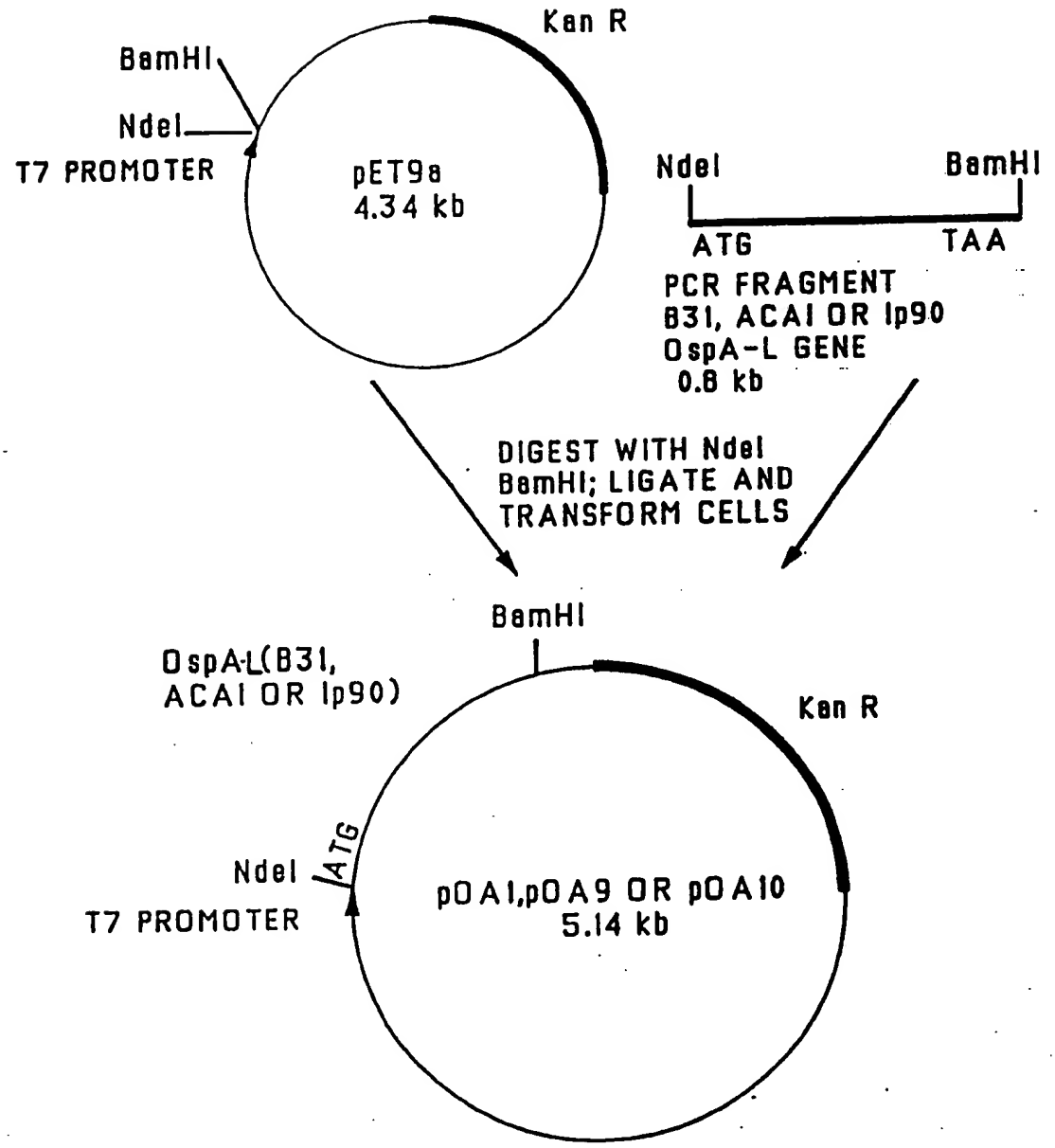
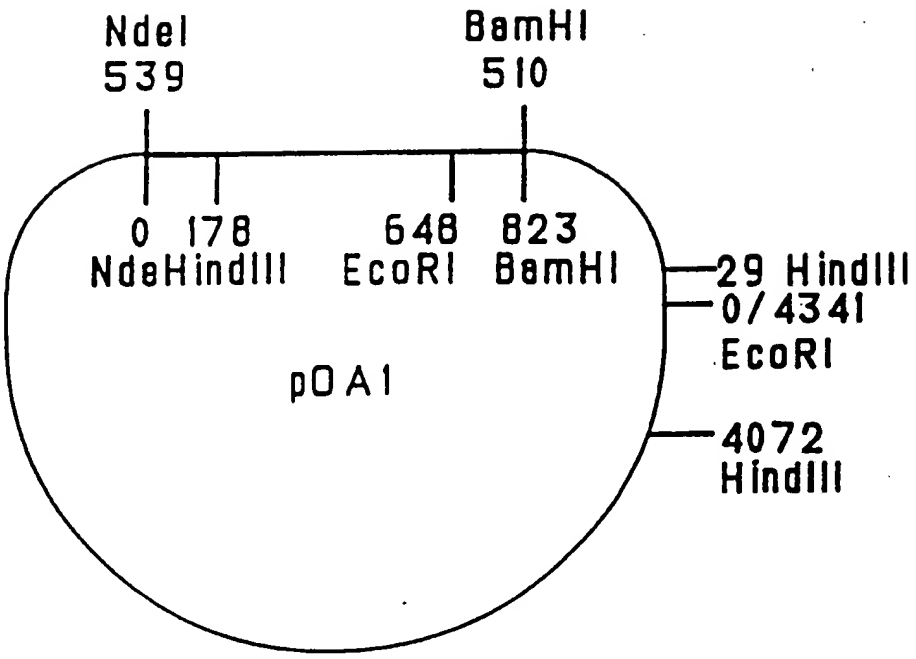
5'-GGCCGCACATATGAAAAAATATTTATTGGG-3' OspN2
NdeI
Ip90
BamHI
3'-CTACGAAATTTTATCCTAGGGGC-5' PK4
5'-ATATATTATGAAAAAATATTTATT.....AACTTAAAGATGCTTTAAAATAGGGAG-3'
3'-TATATAATACTTTTTTATAAATAA..... TTGAATTTCTACGAAATTTTATCCCTC-5
5'-GGCCGCACATATGAAAAAATATTTATTGGG-3' OspN2
NdeI

FIG.1.



pOA1 =PCR FRAGMENT OF B31 OspA GENE IN pET9a
pOA9 =PCR FRAGMENT OF AcaL OspA GENE IN pET9a
pOA10 =PCR FRAGMENT OF IP90 OspA GENE IN pET9a

FIG.2.



PREDICTED FRAGMENTS (bp)

EcoRI	4450	HindIII	3711	NdeI+BamHI	4312
	685		1126		823
			298		
HindIII+NdeI	3533				
	1126				
	298				
	178				

FIG. 3.

Restriction Digest of pOA1

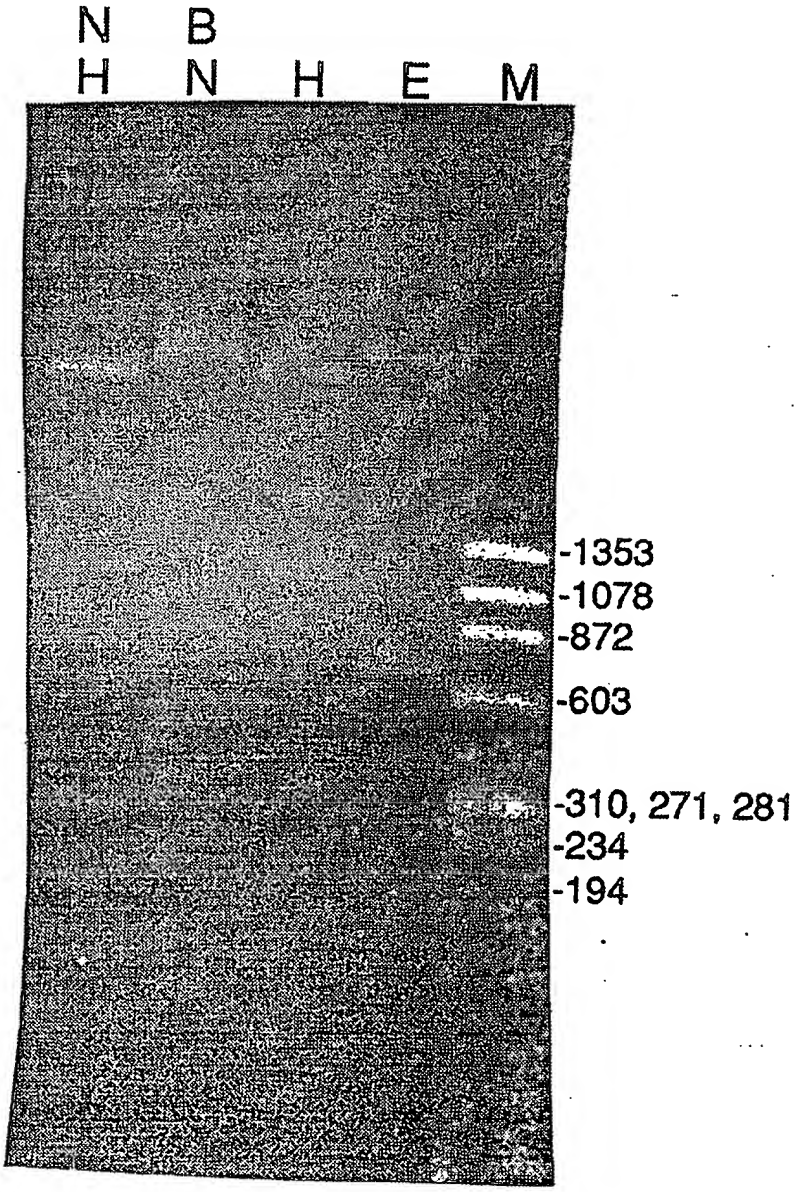


FIG. 4.

PCR Oligos for Cloning into pCMB1 Expression Vector

FIG.5.

B31

BamHI

3'-GCGAAATTTTATTCCTCCCTAGGGGC-5' CO3

5'-ATATATTATGAAAAAATATTTATTGGGAATAGGTCT.....AAAAACGCTTTAAAATAAGGAG-3'

3'-TATATAATACTTTTTTATAAATAACCCTTATCCAGA.....TTTTTGCGAAATTTTATTCCTC-5'

5'-CGGCCATGGAAAAATATTTATTGGG-3' PK3

NcoI

ACAI

BamHI

3'-GCGAAACTTTATTTATTCCCCTAGGGGC-5' BZ1

5'-ATATATTATGAAAAAATATTTATTGGGAATAGGTCT.....AACTTAAAAACGCTTTGAAATAAATAAGGAG-3'

3'-TATATAATACTTTTTTATAAATAACCCTTATCCAGA.....TTGAATTTTTGCGAAACTTTATTTATTCCTC -5'

5'-CGGCCATGGAAAAATATTTATTGGG-3' PK3

NcoI

IP90

BamHI

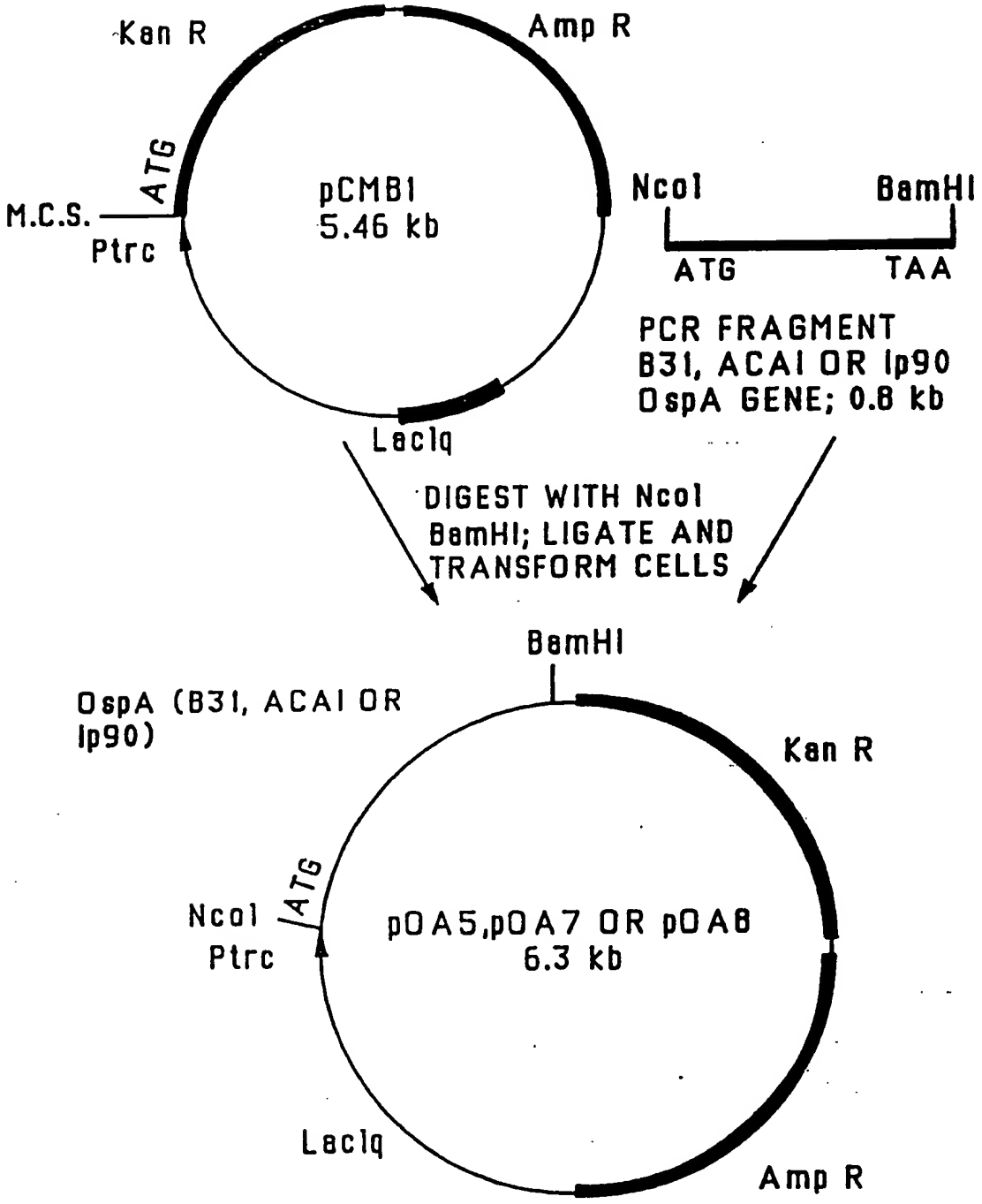
3'-CTACGAAATTTTATCCCTAGGGGC-5' PK4

5'-ATATATTATGAAAAAATATTTATTGGGAATAGGTCT.....AACTTAAAGATGCTTTAAAATAGGGAG-3'

3'-TATATAATACTTTTTTATAAATAACCCTTATCCAGA..... ..TTGAATTTCTACGAAATTTTATCCCTC-5'

5'-CGGCCATGGAAAAATATTTATTGGG-3' PK3

NcoI



pOA5 = PCR FRAGMENT OF B31 OspA GENE IN pCMB1
pOA7 = PCR FRAGMENT OF AceL OspA GENE IN pCMB1
pOA8 = PCR FRAGMENT OF IP90 OspA GENE IN pCMB1

FIG.6.

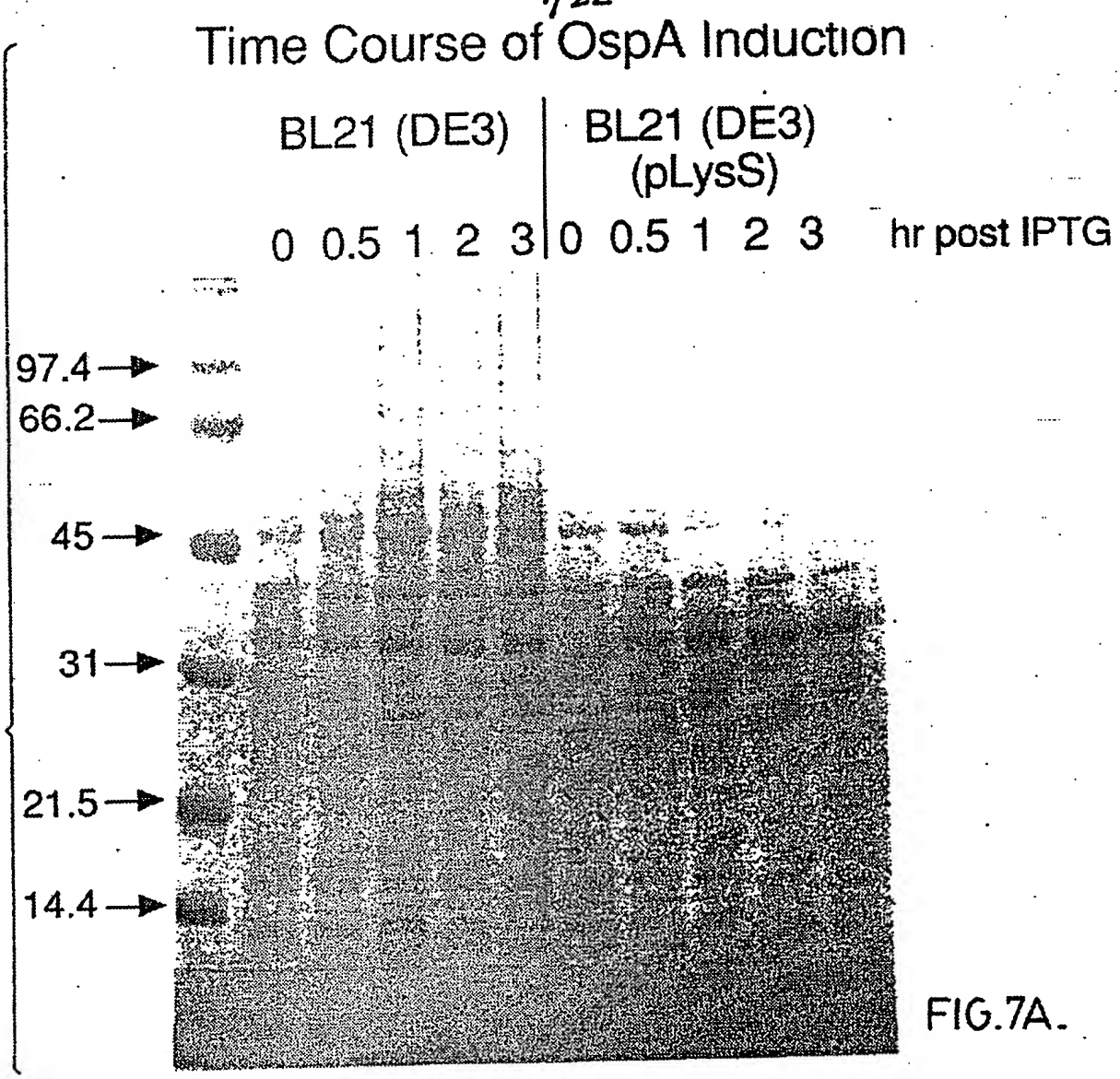


FIG.7A.

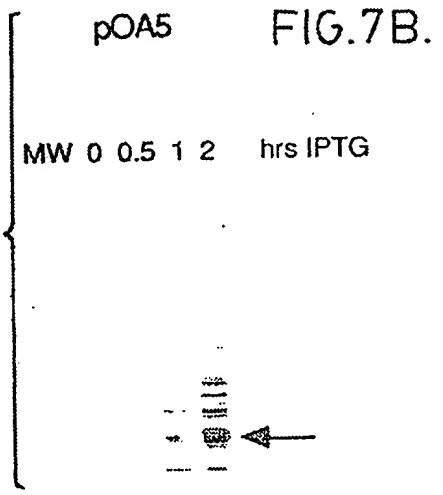


FIG.7B.

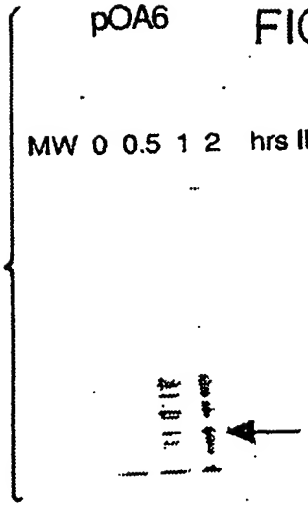


FIG.7C.

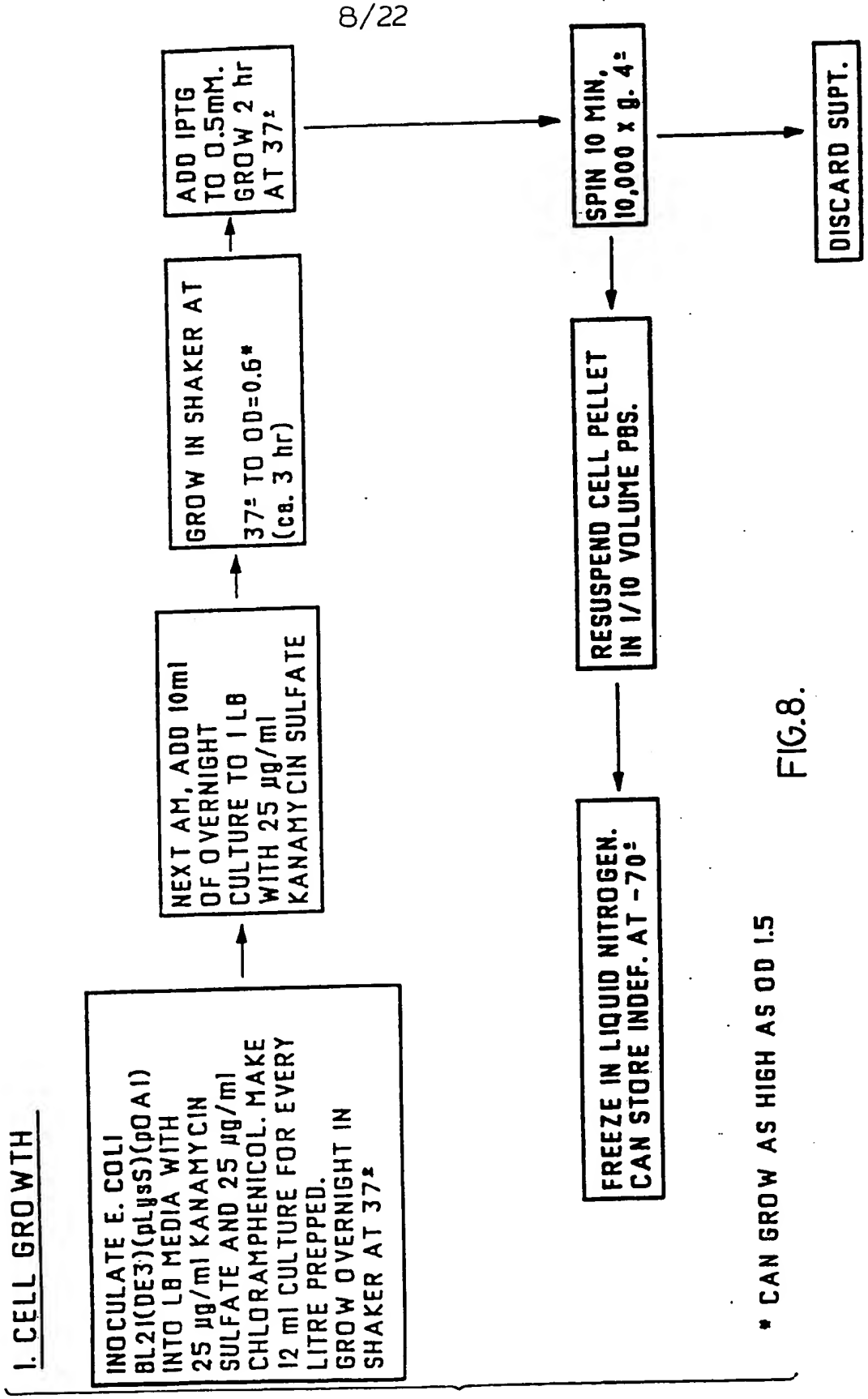

1. CELL GROWTH
INOCULATE E. COLI BL21(DE3)(pLysS)(pOA1) INTO LB MEDIA WITH 25 µg/ml KANAMYCIN SULFATE AND 25 µg/ml CHLORAMPHENICOL. MAKE 12 ml CULTURE FOR EVERY LITRE PREPPED. GROW OVERNIGHT IN SHAKER AT 37°
NEXT AM, ADD 10ml OF OVERNIGHT CULTURE TO 1 LB WITH 25 µg/ml KANAMYCIN SULFATE
GROW IN SHAKER AT 37° TO OD=0.6* (ca. 3 hr)
ADD IPTG TO 0.5mM. GROW 2 hr AT 37°
SPIN 10 MIN, 10,000 x g. 4°
DISCARD SUPT.
RESUSPEND CELL PELLET IN 1/10 VOLUME PBS.
FREEZE IN LIQUID NITROGEN. CAN STORE INDEF. AT -70°
* CAN GROW AS HIGH AS OD 1.5

FIG.8.

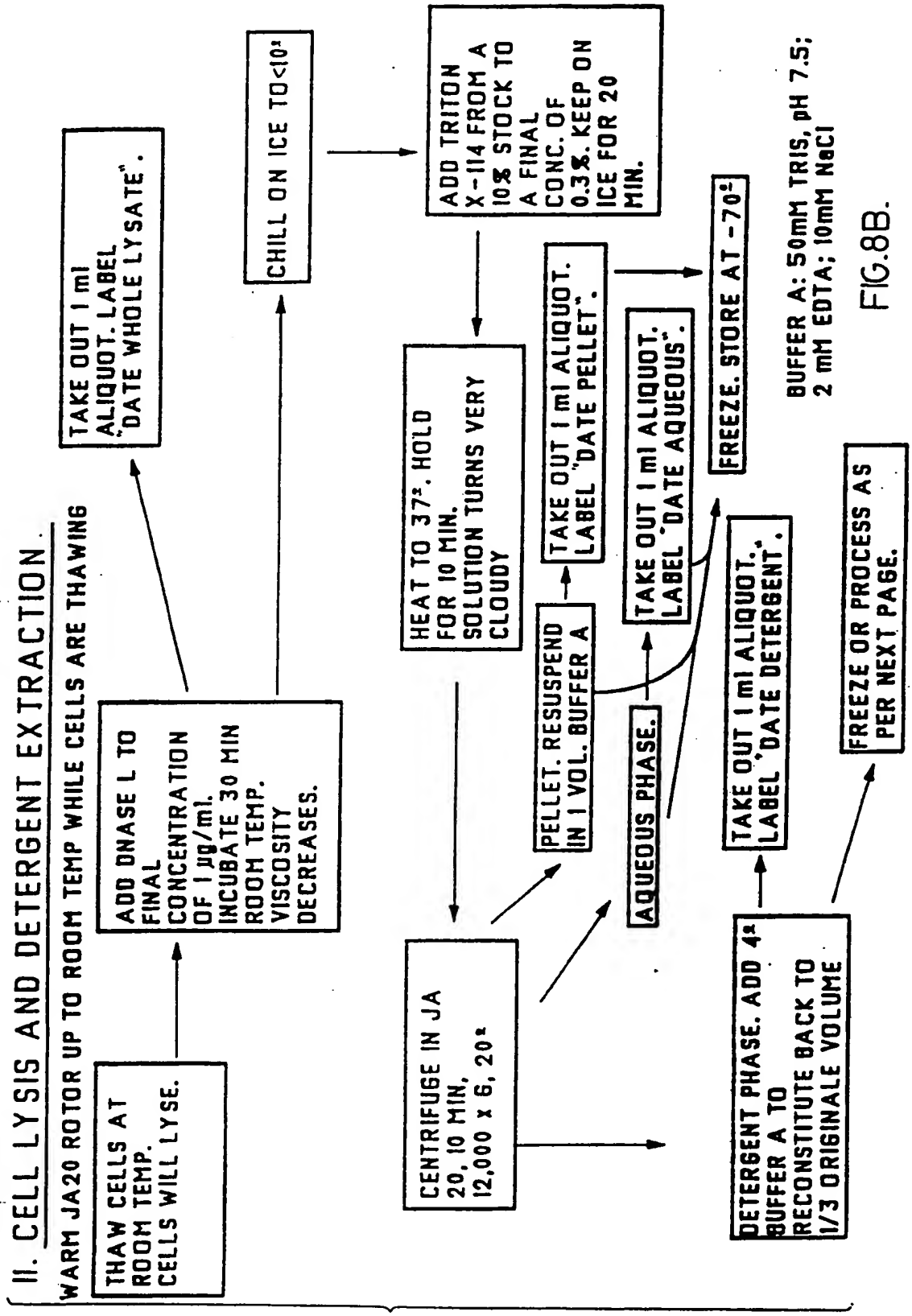

II. CELL LYSIS AND DETERGENT EXTRACTION
WARM JA20 ROTOR UP TO ROOM TEMP WHILE CELLS ARE THAWING
THAW CELLS AT ROOM TEMP. CELLS WILL LYSE.
ADD DNASE L TO FINAL CONCENTRATION OF 1 µg/ml. INCUBATE 30 MIN ROOM TEMP. VISCOSITY DECREASES.
TAKE OUT 1 ml ALIQUOT. LABEL "DATE WHOLE LYSATE".
CHILL ON ICE TO<10°
ADD TRITON X-114 FROM A 10% STOCK TO A FINAL CONC. OF 0.3%. KEEP ON ICE FOR 20 MIN.
HEAT TO 37°. HOLD FOR 10 MIN. SOLUTION TURNS VERY CLOUDY
CENTRIFUGE IN JA 20, 10 MIN, 12,000 x 6, 20°
PELLET. RESUSPEND IN 1 VOL. BUFFER A
TAKE OUT 1 ml ALIQUOT. LABEL "DATE PELLET".
AQUEOUS PHASE.
TAKE OUT 1 ml ALIQUOT. LABEL "DATE AQUEOUS".
FREEZE. STORE AT -70°
DETERGENT PHASE. ADD 4° BUFFER A TO RECONSTITUTE BACK TO 1/3 ORIGINALE VOLUME
TAKE OUT 1 ml ALIQUOT. LABEL "DATE DETERGENT".
FREEZE OR PROCESS AS PER NEXT PAGE.
BUFFER A: 50mM TRIS, pH 7.5; 2 mM EDTA; 10mM NaCl

FIG.8B.

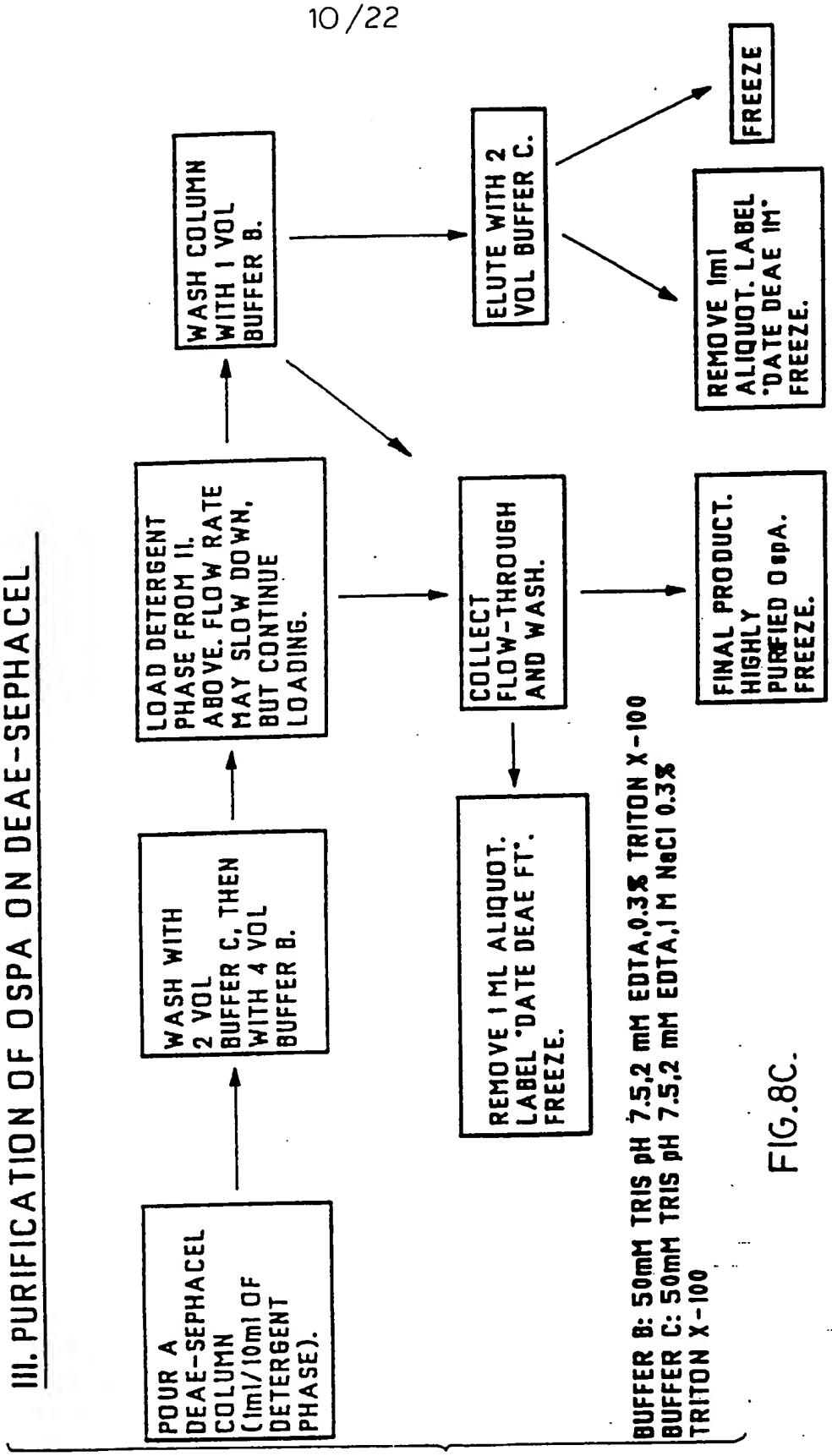

III. PURIFICATION OF OSPA ON DEAE-SEPHACEL
POUR A DEAE-SEPHACEL COLUMN (1ml/10ml OF DETERGENT PHASE).
WASH WITH 2 VOL BUFFER C, THEN WITH 4 VOL BUFFER B.
LOAD DETERGENT PHASE FROM II. ABOVE. FLOW RATE MAY SLOW DOWN, BUT CONTINUE LOADING.
WASH COLUMN WITH 1 VOL BUFFER B.
ELUTE WITH 2 VOL BUFFER C.
FREEZE
REMOVE 1ml ALIQUOT. LABEL "DATE DEAE 1M" FREEZE.
COLLECT FLOW-THROUGH AND WASH.
FINAL PRODUCT. HIGHLY PURIFIED OspA. FREEZE.
REMOVE 1 ML ALIQUOT. LABEL "DATE DEAE FT". FREEZE.
BUFFER B: 50mM TRIS pH 7.5,2 mM EDTA,0.3% TRITON X-100
BUFFER C: 50mM TRIS pH 7.5,2 mM EDTA,1 M NaCl 0.3% TRITON X-100

FIG.8C.

Selective Solubilization of OspA by Triton X-114

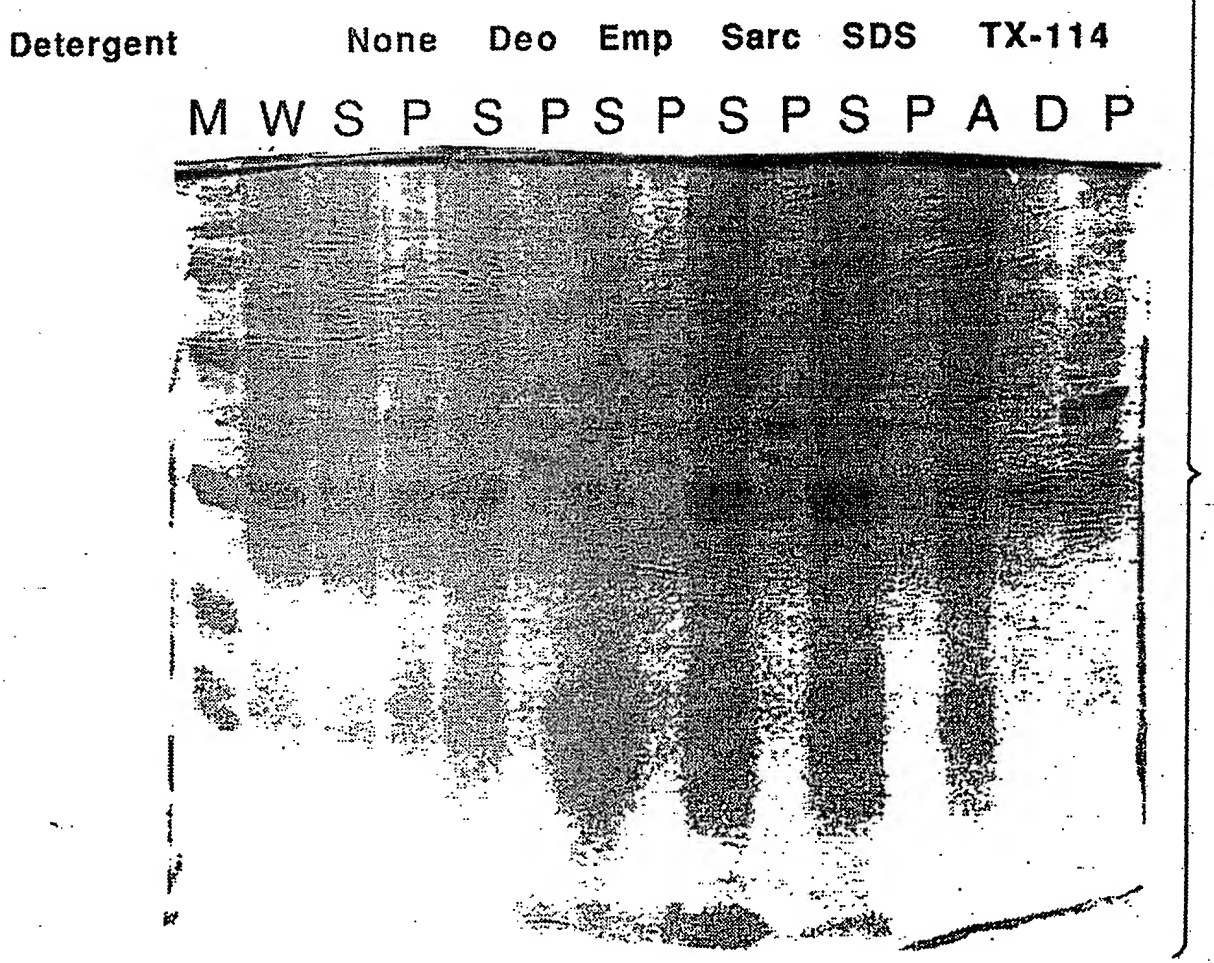


FIG.9.

Western Blot of Purification Fractions with Mab H5332

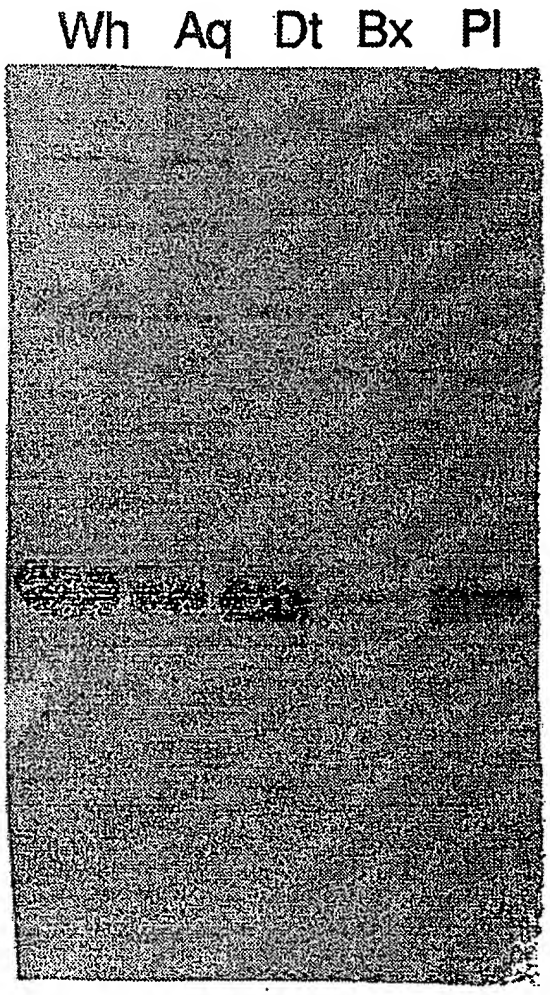


FIG. 10.

FIG.11.

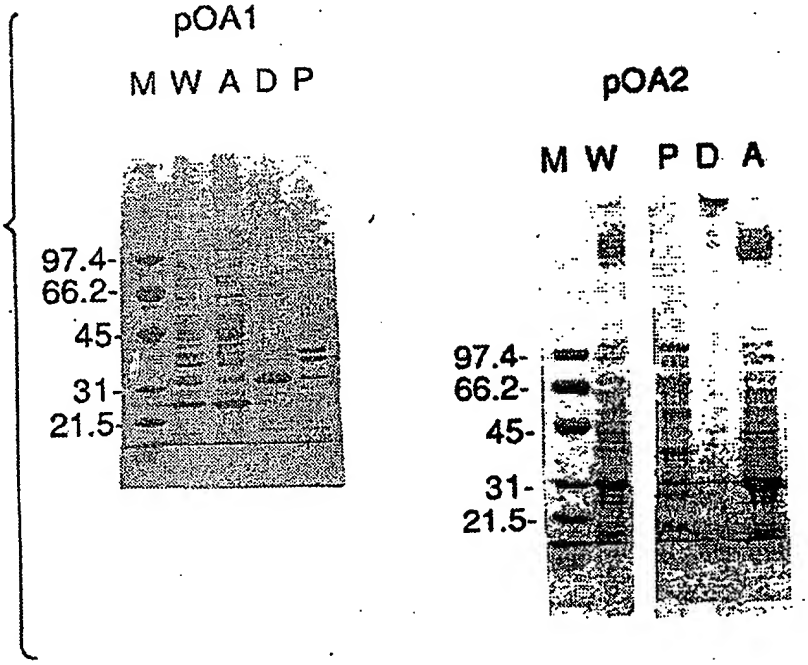


FIG.12A.

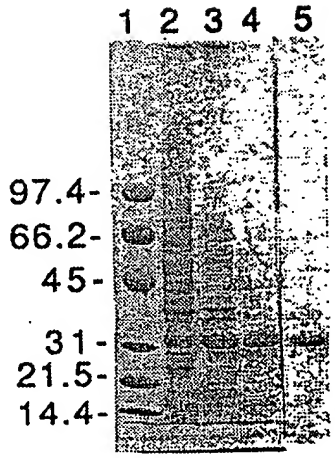


FIG.12B.

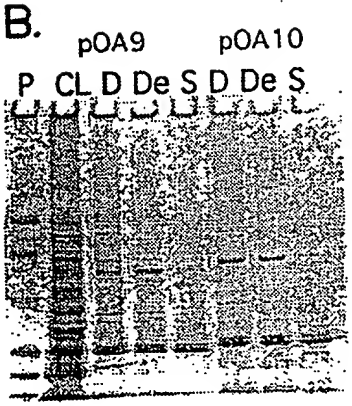
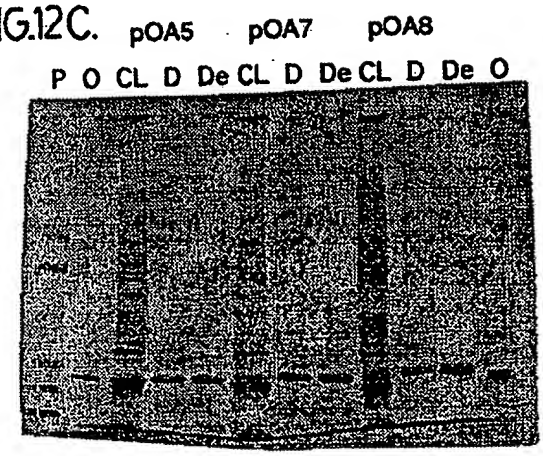


FIG.12C.



Immunogenicity of Recombinant OspA vs. Borrelia Protein

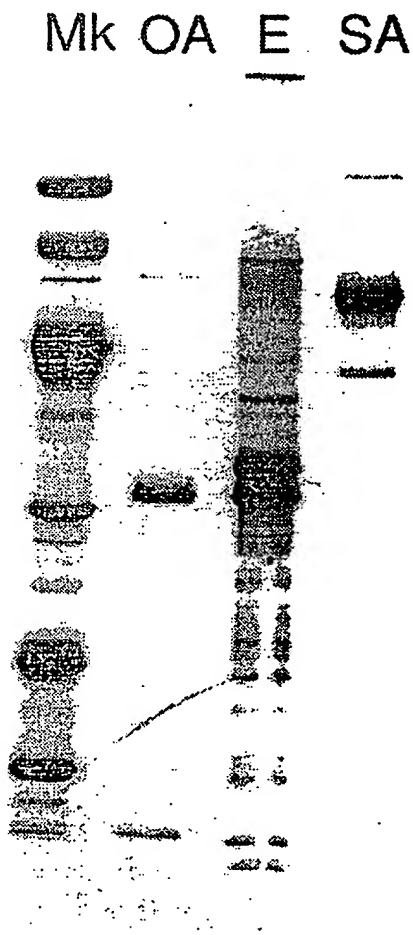


FIG. 13.

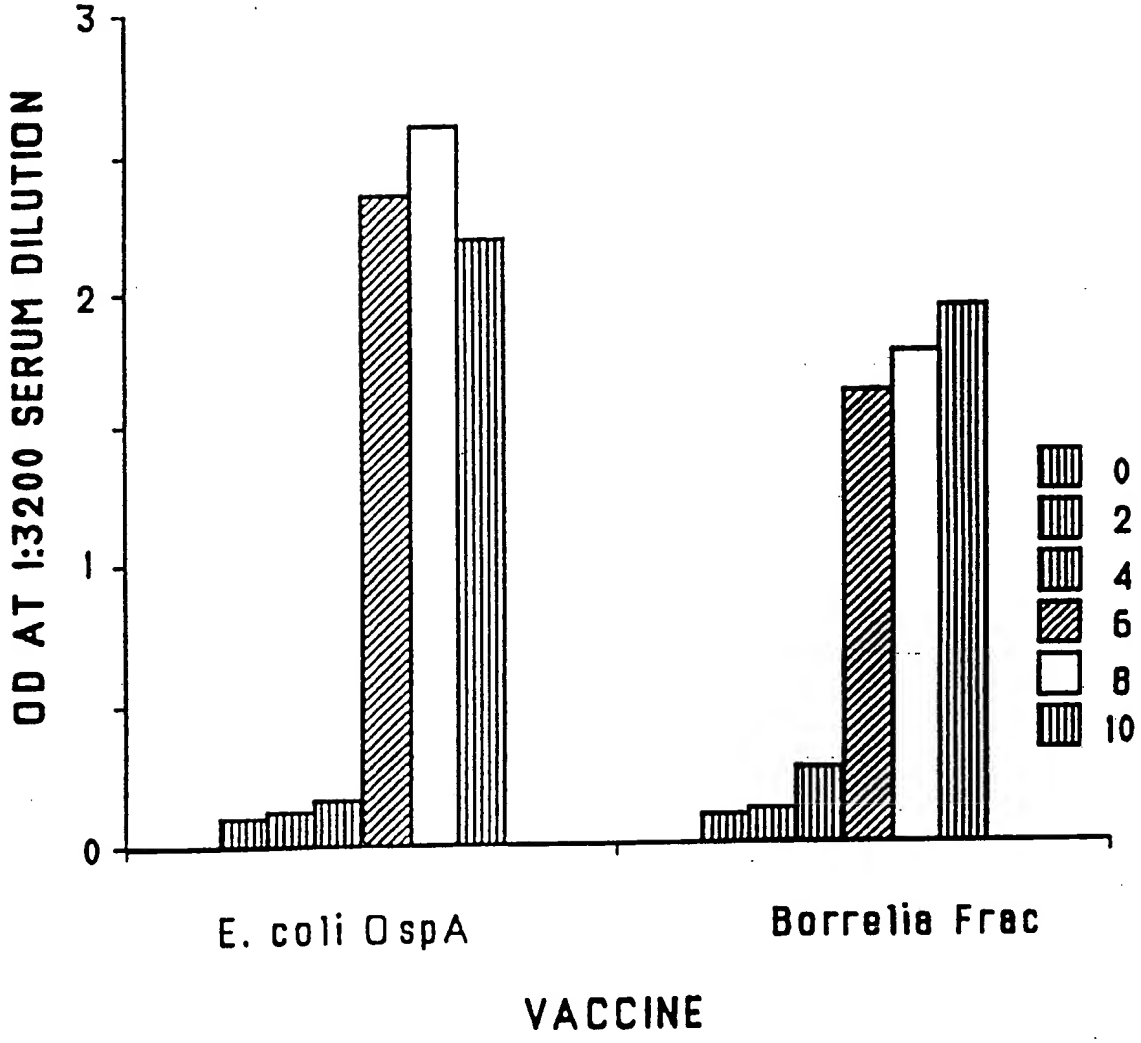

OD AT 1:3200 SERUM DILUTION
3
2
1
0
0
2
4
6
8
10
E. coli OspA
Borrelia Frac
VACCINE

FIG. 14.

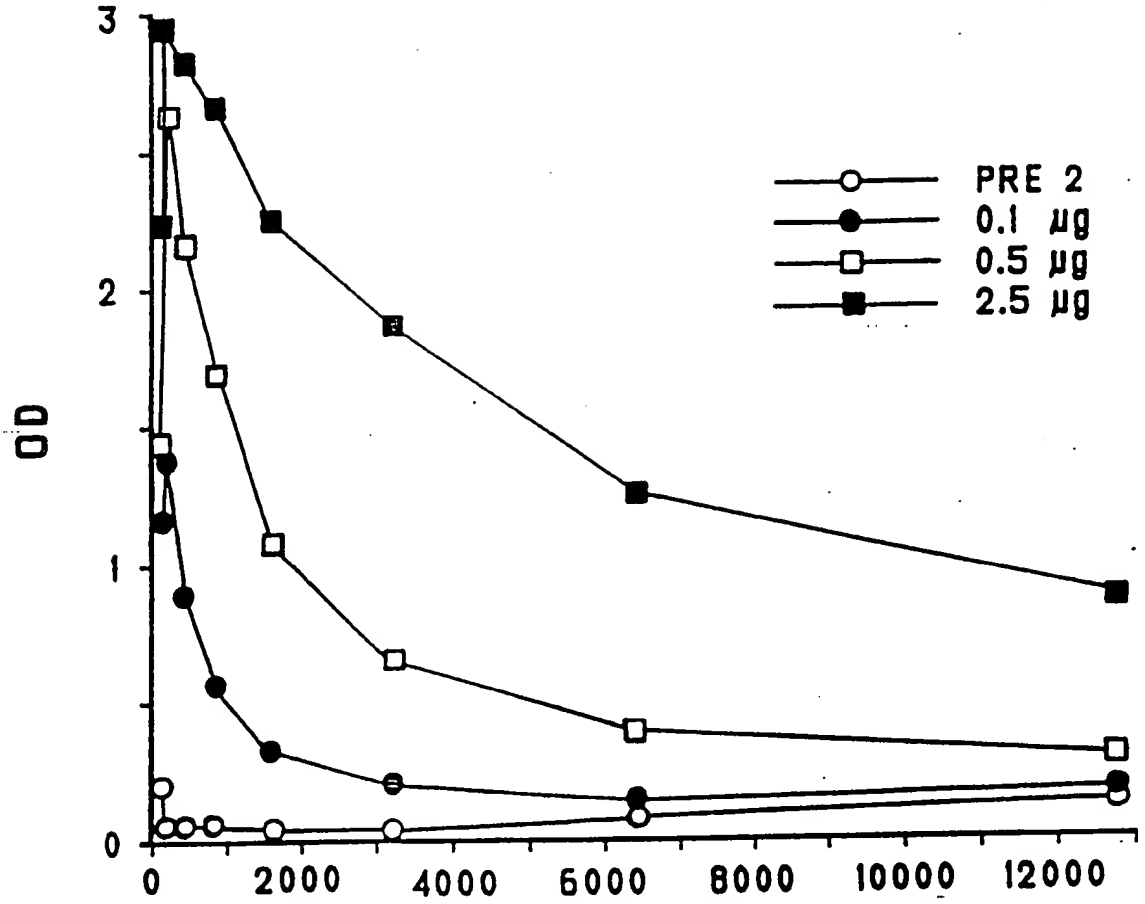

3
2
1
0
OD
0
2000
4000
6000
8000
10000
12000
SERUM DIL
PRE 2
0.1 µg
0.5 µg
2.5 µg

FIG.15A.

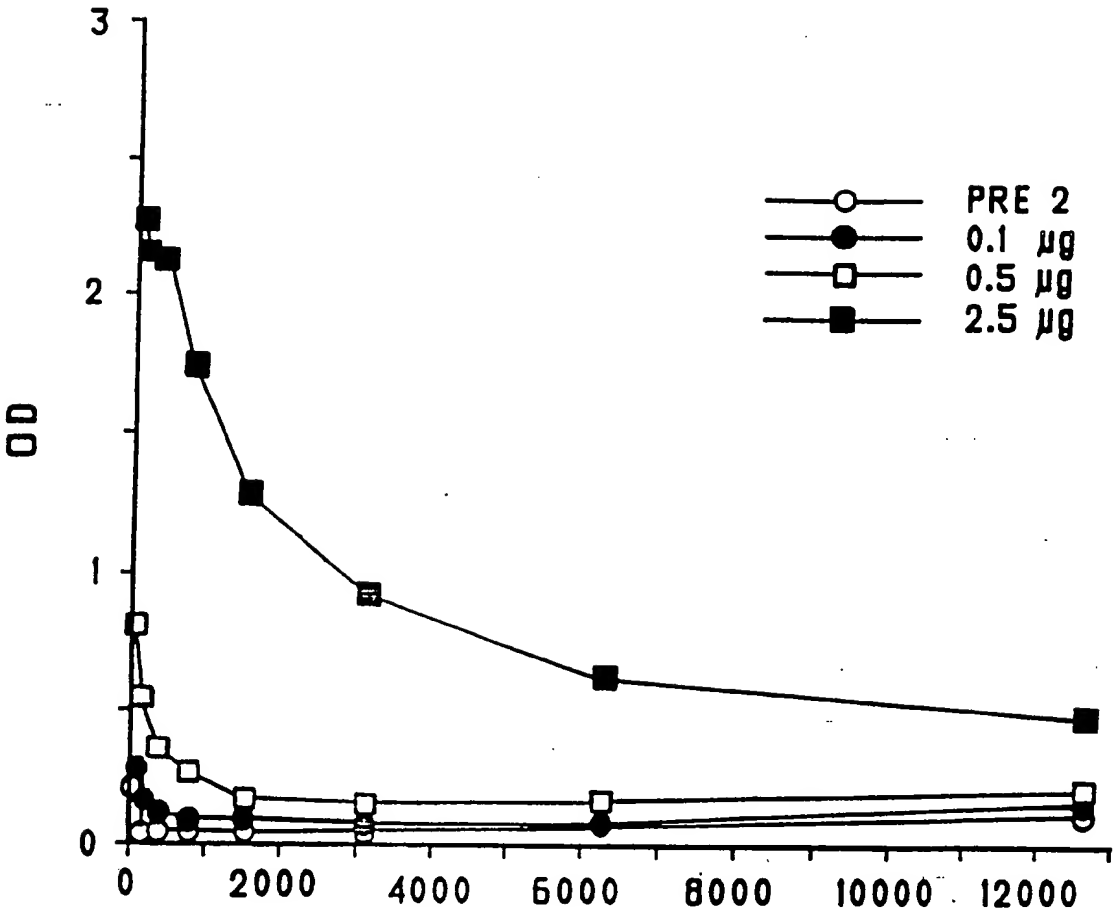

3
2
1
0
OD
0
2000
4000
6000
8000
10000
12000
PRE 2
0.1 µg
0.5 µg
2.5 µg
SERUM DIL

FIG. 15 B.

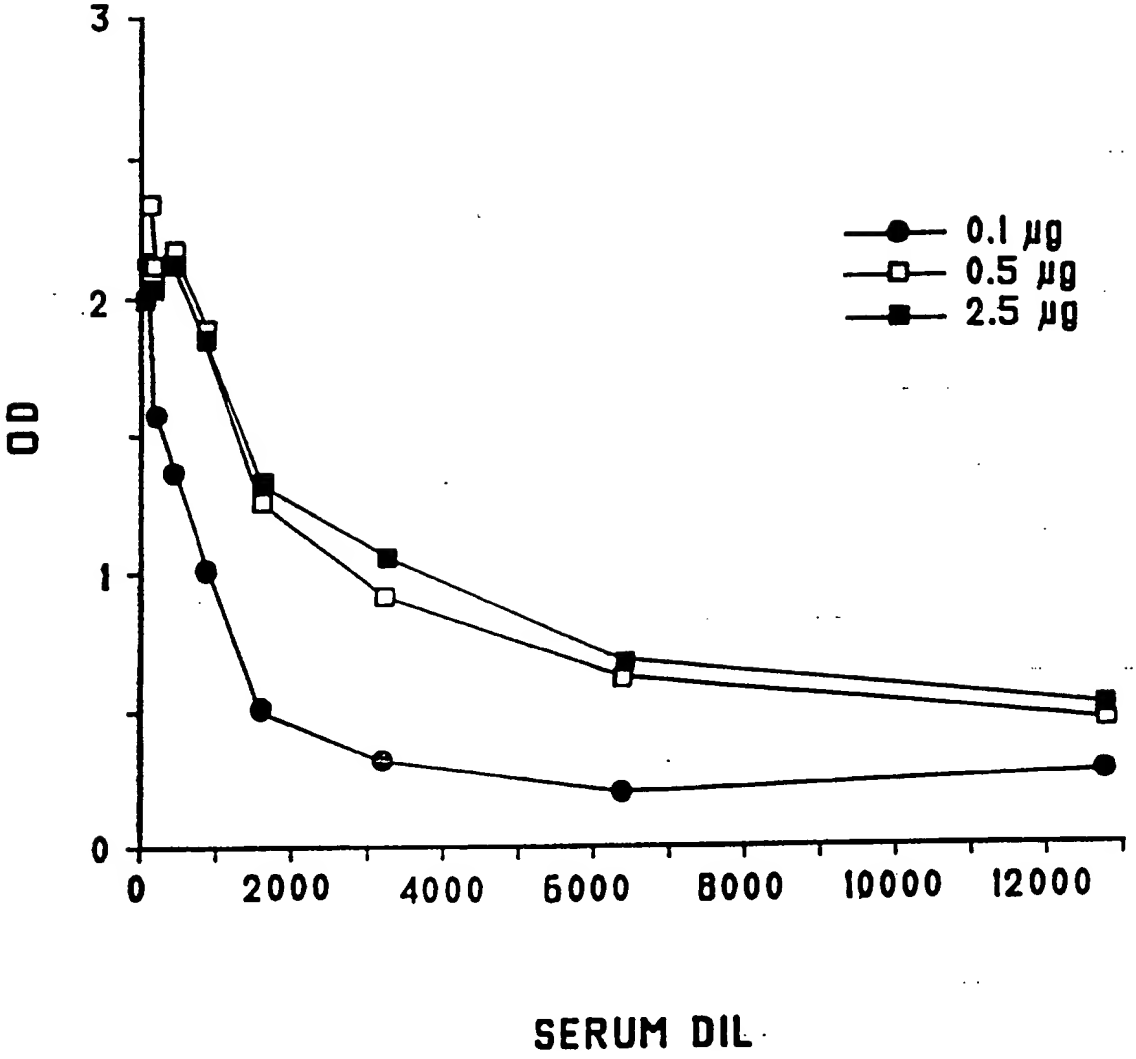

3
2
1
0
OD
0
2000
4000
6000
8000
10000
12000
SERUM DIL
0.1 µg
0.5 µg
2.5 µg

FIG.15C.

FIG. 15D(2)

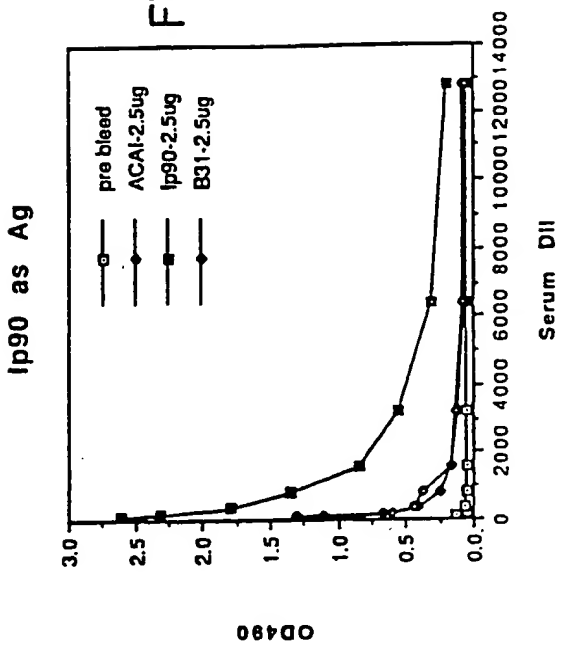


FIG. 15D(1)

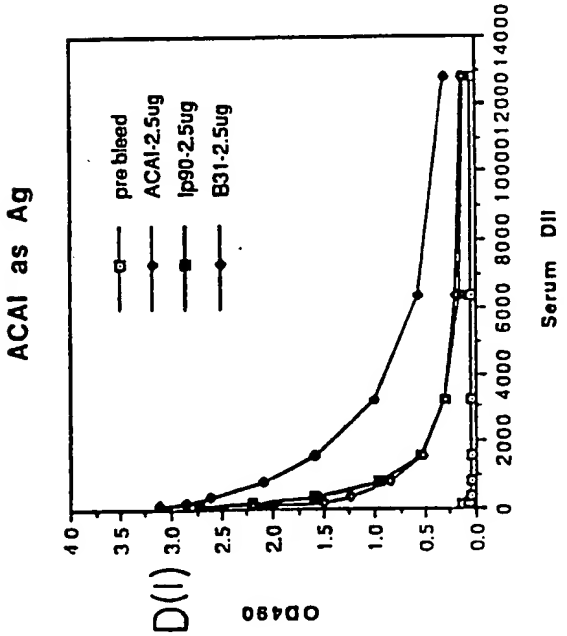
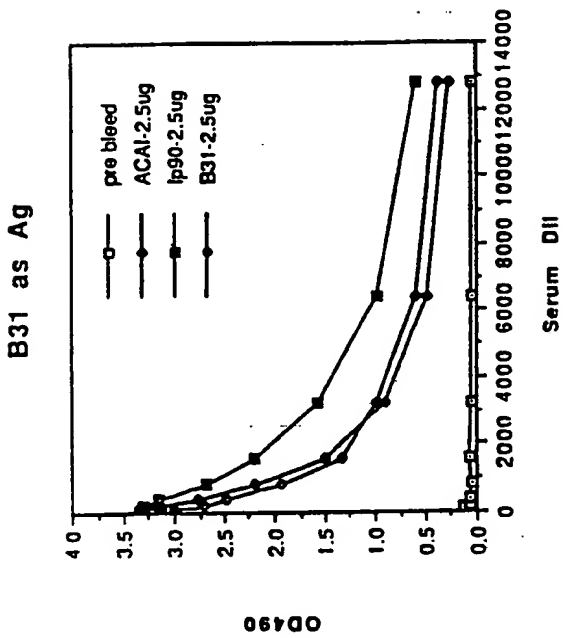


FIG. 15D(3)



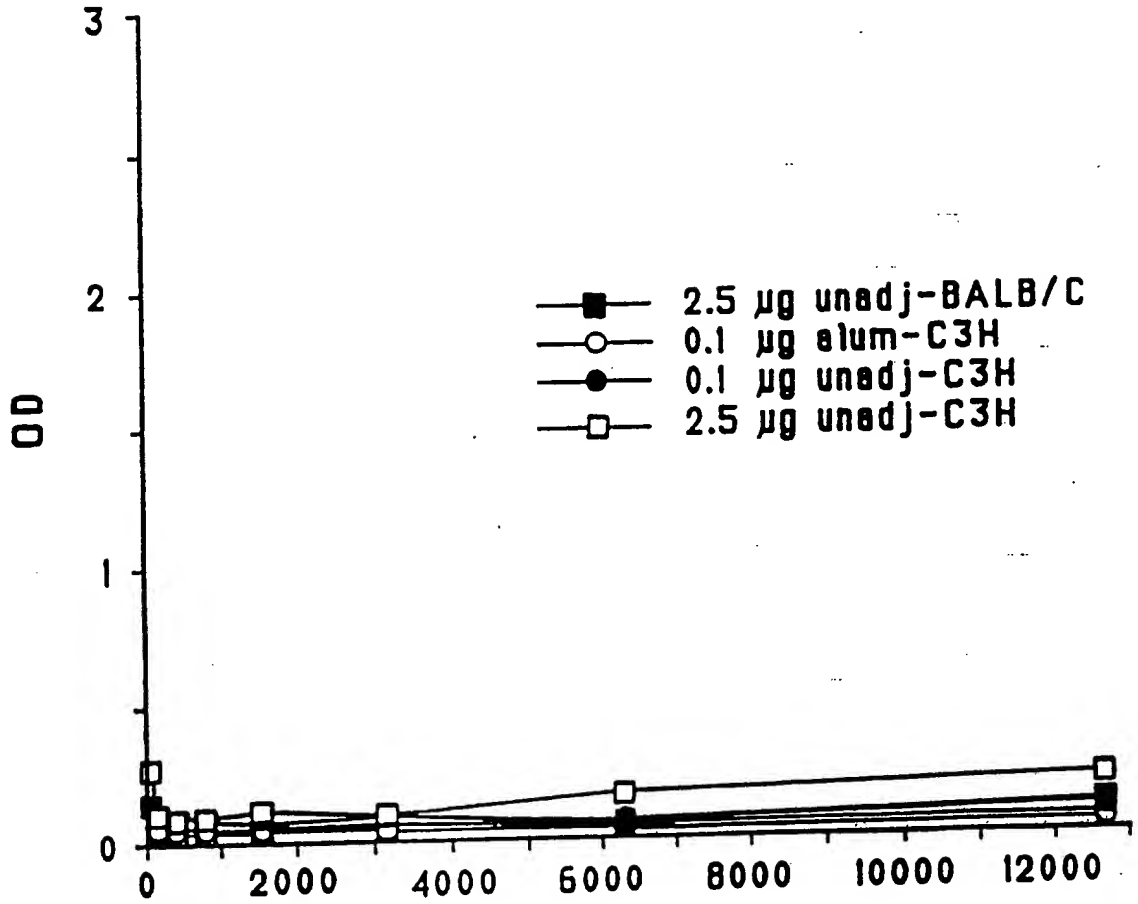

3
2
1
0
OD
0
2000
4000
6000
8000
10000
12000
SERUM DIL
2.5 µg unadj-BALB/C
0.1 µg alum-C3H
0.1 µg unadj-C3H
2.5 µg unadj-C3H

FIG.15E.

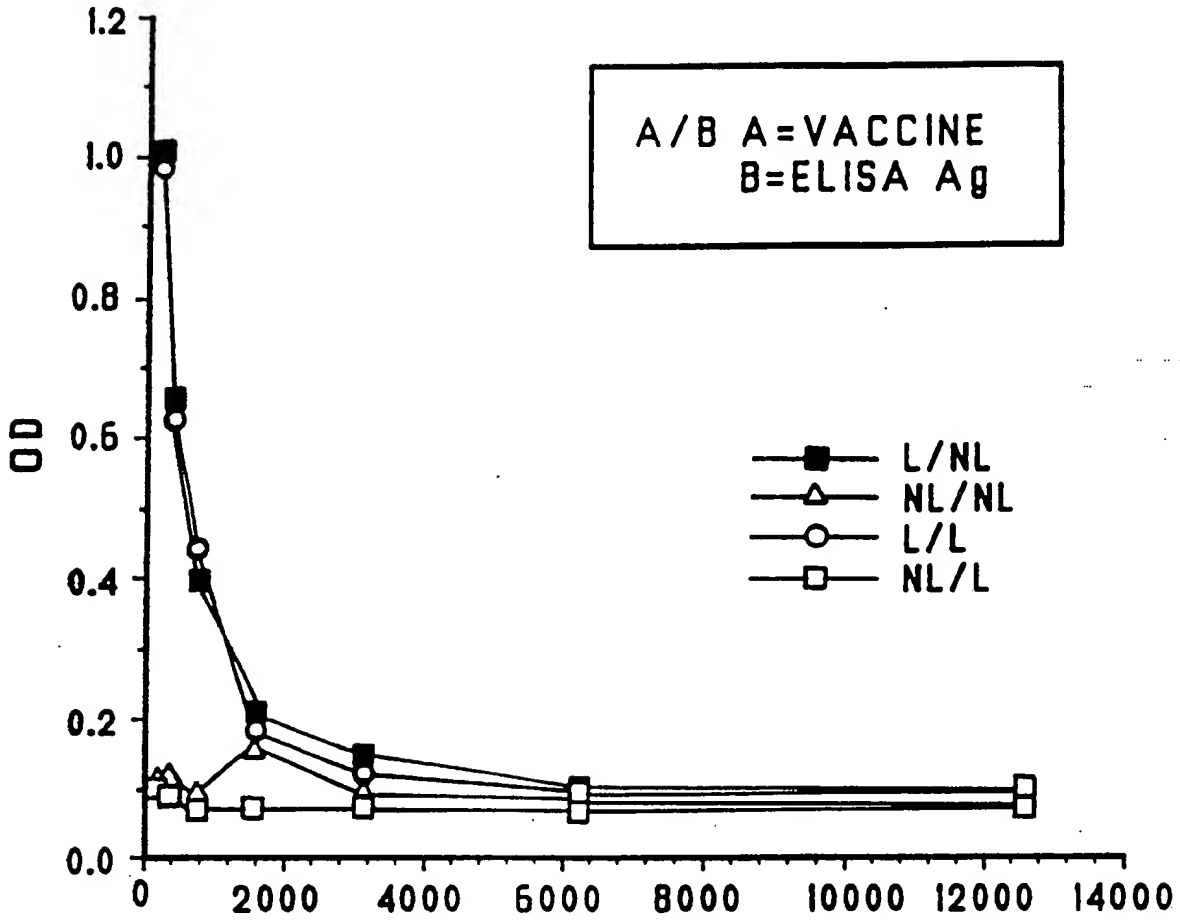

A/B A=VACCINE
B=ELISA Ag
L/NL
NL/NL
L/L
NL/L
OD
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2000
4000
6000
8000
10000
12000
14000
SERUM DILUTION

FIG.16.

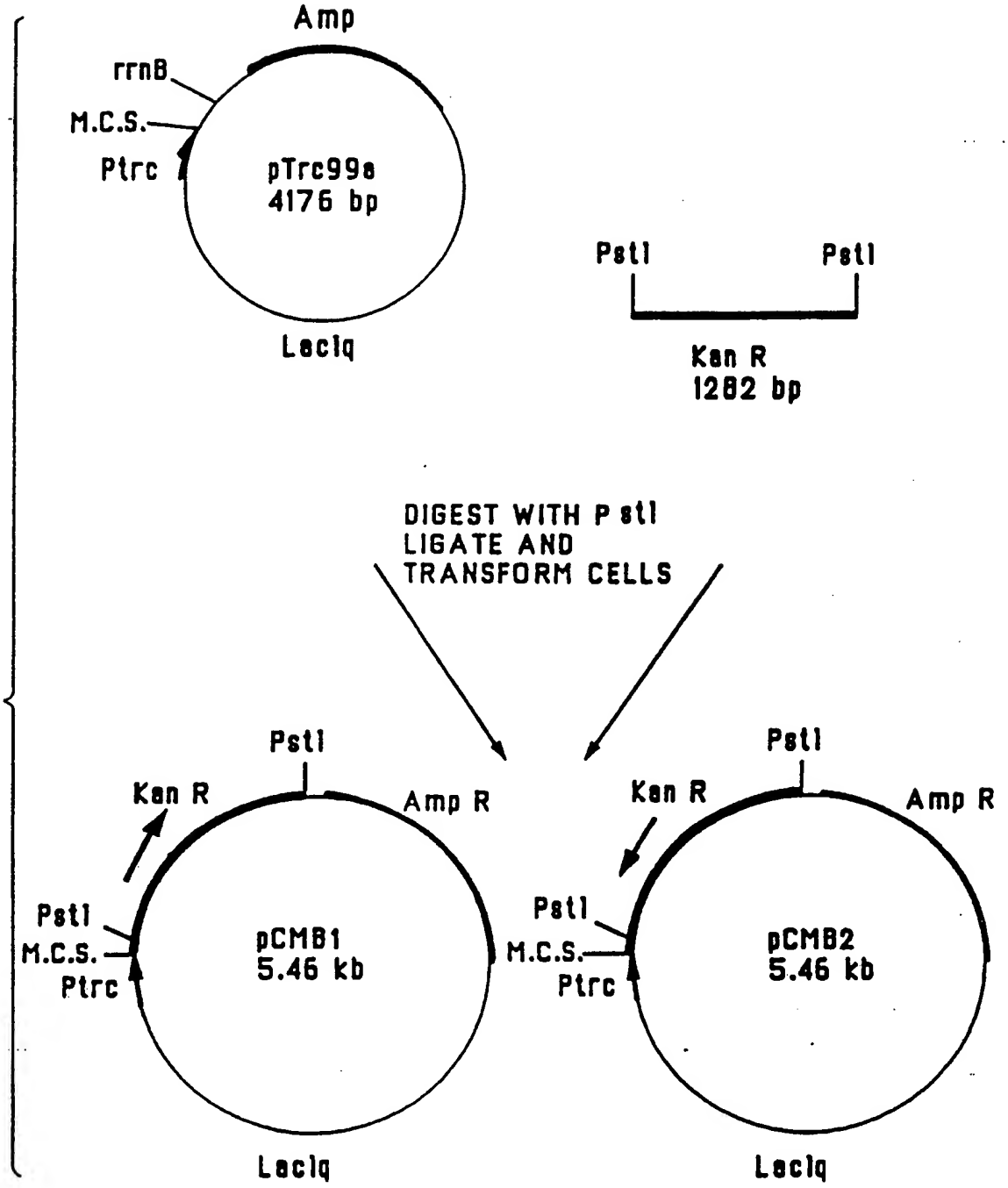

Amp
rrnB
M.C.S.
Ptrc
pTrc99a
4176 bp
LacIq
PstI
PstI
Kan R
1282 bp
DIGEST WITH P stI
LIGATE AND
TRANSFORM CELLS
PstI
Kan R
Amp R
PstI
M.C.S.
Ptrc
pCMB1
5.46 kb
LacIq
PstI
Kan R
Amp R
PstI
M.C.S.
Ptrc
pCMB2
5.46 kb
LacIq

FIG.17.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08697

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/06; C12Q 1/00; C07K 3/00; A61K 39/00
US CL :435/69.3, 7.1; 530/350, 806; 424/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.3, 7.1; 530/350, 806; 424/88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS, GENBANK
search terms: ospA, Borrelia, lipoprotein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Chromatography, Volume 521, No. 2, issued 23 November 1990, K.B. Gondolf et al, "Isolation of an outer membrane protein complex from Borrelia burgdorferi by n-butanol extraction and high-performance ion-exchange chromatography", pages 325-334, see Figure 4.	1-5,10,13-18, 28-31 6-9,11,12
Y	The Journal of Infectious Diseases, Volume 152, No. 3, issued September 1985, A.G. Barbour et al, "Heterogeneity of major proteins in Lyme disease Borrelia: a molecular analysis of North American and European isolates", pages 478-484, see entire document.	6-9,11,12
Y	Nucleic Acids Research, Volume 17, No. 21, issued 1989, R. Wallich et al, "Cloning and sequencing of the gene encoding the outer surface protein (OspA) of a European Borrelia burgdorferi isolate", page 8864, entire document.	1-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

05 JANUARY 1993

Date of mailing of the international search report

15 JAN 1993

Name and mailing address of the ISA/
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Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08697

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Protein Expression and Purification, Volume 1, issued November 1990, J.J. Dunn et al, "Outer surface protein A (OspA) from the Lyme disease spirochete, Borrelia burgdorferi; high level expression and purification of a soluble recombinant form of OspA", pages 159-168, see Materials and Methods, page 167, last paragraph. and Figures 4-6.	1-5,10,13-18,28-31 6-9,11,12,23-24
X Y	The Journal of Infectious Diseases, Volume 164, No.1, issued July 1991, M.M. Simon et al, "Recombinant outer surface protein A from Borrelia burgdorferi induces antibodies protective against spirochetal infection in mice", pages 123-132, see entire document, especially Figures 1 and 4.	1-5,10,13-18,28-34 6-9,11,12
X Y	Infection and Immunity, Volume 58, No. 4, issued April 1990, M.E. Brandt et al, "Immunogenic integral membrane proteins of Borrelia burgdorferi are lipoproteins", pages 983-991, see Material and Methods and Figures 1-2.	1-5,10,13-18, 28-31 6-9,11,12,19-22,25-27
Y	Infection and Immunity, Volume 56, NO. , issued February 1988, J.D. Radolf et al, "Identification and localization of integral membrane proteins of virulent Treponema pallidum subsp. pallidum by phase partitioning with nonionic detergent Triton X-114", pages 490-498, see Materials and Methods.	19-27
Y	Science, Volume 250, No. 4980, issued 26 October 1990, E. Fikrig et al, "Protection of mice against the Lyme disease agent by immunizing with recombinant OspA", pages 553-556, see page 553.	28-34
Y	Infection and Immunity, Volume 54, No. 1, issued October 1986, T.R. Howe et al, "Organization of genes encoding two outer membrane proteins of the Lyme disease agent Borrelia burgdorferi within a single transcriptional unit", pages 207-212, see entire document.	1-34